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RESTORATION OF SCARRED VOCAL FOLDS WITH STEM CELL IMPLANTATION

- ANALYSES IN A XENOGRAFT MODEL

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In memoriam **Åke Dahlqvist**

Rabbits can grunt, hiss and squeak.
They can also scream but you never want to hear it,
because it means they are really frightened.
Humans can do the same and you still do not want to hear it,
because it can cause scars and you have no treatment

ABSTRACT

Background: Tissue defects in the vocal fold (VF) caused by trauma, surgical procedures, post radiotherapy, often heal with scar formation. The scar tissue causes stiffness of the lamina propria rendering disturbed viscoelastic properties to the VF. A scarred VF causes severe voice problems. Treatment is difficult and presently there is no treatment that heals VF scars.

Objectives: The aims of this thesis were to evaluate if human stem cell transplantations have the potential to heal scarred VFs, and if transplanted stem cells regenerate lost tissue in scarred VFs.

Study Design: Experimental xenograft model.

Methods: The VFs of New Zealand rabbits were scarred by a 1.5–2 mm resection. Human embryonic (hESCs) or mesenchymal (hMSCs) stem cells were thereafter transplanted into the VFs. Analyses for grade of scaring, lamina propria (Lp) thickness, relative content of collagen type I, elastic and dynamic viscoelasticity (G', η'), fluorescence in situ hybridisation for detection of human cells, Verhoeff staining for detection of elastin, alcian blue staining for detection of hyaluronic acid, RT-PCR species-specific analysis for collagen type I, and for Nanog expression of pluripotency were performed.

Results: Both transplanted hESCs (G') (η') and hMSCs (G') significantly improved the viscoelastic properties of the VFs, analyzed at one month. HMSCs also significantly reduced collagen type I content. At three months hMSC treatment significantly reduced both (G') (η') and showed no significant differences to normal VFs. Also collagen type I content and Lp thickness were significantly reduced and were not significantly higher than in normal VFs. A clinic like setting was studied, where VFs were resected and left to spontaneously heal for 9 weeks. The established scar was thereafter excised, hMSCs transplanted in the wound, and the VFs were analyzed after another 10 weeks. The hMSC transplanted VFs showed no significant difference in η', nor in G', nor in Lp-thickness compared to normal VFs, and all three parameters were significantly reduced compared with untreated scarred VFs.

The transplanted hESCs showed after one month, by differentiation, regeneration of epithelium, muscle, cartilage and gland tissue in contact with corresponding rabbit native tissue. HMSCs did not differentiate or regenerate tissue. Pluripotent hESCs were shown to survive one month, and hMSCs survived four but not ten weeks in the rabbit VFs. The transplanted VFs showed no malignancy or teratoma formation.

The immunosuppressant Tacrolimus used to reduce the host versus graft reaction suppressed the ant-scaring effect of the transplanted hMSCs.

Conclusion: Both human embryonic and mesenchymal stem cells have the potential to improve healing and to restore the rheological properties of scarred VFs. Human embryonic stem cells transplanted into scarred rabbit vocal folds differentiate and regenerate human tissue compatible with the surrounding native tissue.

LIST OF PUBLICATIONS

I. Hertegard S, Cedervall J, **Svensson B,** Forsberg K, Maurer F.H.J, Vidovska D, Olivius P, Ahrlund-Richter L, Le Blanc K. Viscoelastic and histological properties in scarred rabbit vocal folds after mesenchymal stem cell injection. *Laryngoscope* 2006;116(7):1248-1254.

II. Cedervall J, Ährlund-Richter L, **Svensson B**, Forsgren K, Maurer FHJ, Vidovsk D, Hertegård S. Injection of embryonic stem cells into scarred rabbit vocal folds enhances healing and improves viscoelasticity: short-term results. *Laryngoscope* 2007;117:2075-2081.

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LIST OF ABBREVIATIONS

bFGF	Basic fibroblast growth factor
BM	Bone marrow
dLp	Lamina propria deep layer
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESC	Embryonic Stem Cell
FGF	Fibroblast growth factor
FISH	Fluorescence In Situ Hybridization
FbRS	Reinke space fibroblasts
FbMF	Maculae flavae fibroblasts
HE	Hematoxylin & Eosin
hESC	Human embryonic stem cell
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
hMSC	Human mesenchymal stem cell
iLp	Lamina propria intermediate layer
IL-1,4,6,8	Interleukin – 1,4,6,8
Lp	Lamina propria
MSC	Mesenchymal stem cell
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGE-2	Prostaglandin E 2
PGII	Proteoglycan II
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sLp	Lamina propria superficial layer
TC	Immunosuppressant Tacrolimus
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
VFSCs	Vocal fold stellate shaped fibroblast like cells
VF	Vocal Fold

1 BACKGROUND

Scar tissue in a vocal fold (VF) disturbs the delicate balance of viscosity in the superficial layers of the VF. The superficial layers of the VF compose the lamina propria (Lp). Scar tissue causes stiffness of the Lp and this renders disturbed vibratory characteristics to the VF (Hirano and Kakita, 1985; Benninger et al., 1996). A scar formation in the VF causes severe voice problems and the patient becomes breathy or aphonic and the phonation threshold pressure which corresponds to "easiness of phonation" is elevated (Lehman et al., 1988). Surgical procedures or radiation therapy to the VFs often cause scar formation in the Lp and in the superficial part of the thyroarytenoid muscle. Scar formation in a VF is a demanding challenge to treat (Hirano et al., 2009; Thibeault et al., 2003).

Several substances have been used to augment scarred or atrophic VFs, bovine or human collagen (Ford et al., 1992; Remacle et al., 1999), autologous fat (Shindo et al., 1996; Neuenschwander et al., 2001), hyaluronan (Dahlqvist et al., 2004; Hansen et al., 2005; Hertegard et al., 2006;), autologous implantation of fascia (Tsunoda et al., 2005), injection of hepatocyte growth factor (Hirano et al., 2004), and autologous fibroblasts (Chhetri et al., 2004, 2011; Thibeault et al., 2009). Several of these substances have been shown to improve the vibratory characteristics of scarred VFs and indeed speech production, but presently there are no effective methods to prevent or heal VF scars or defects (Mallur and Rosen, 2010).

Stem cells open up a new field of biomechanical research. Stem cells are undifferentiated cells that have the capacity to differentiate into specialized cell types. They also have the capacity to self-renewal, i.e. make more cells like themselves. Stem cells are classified after their ability to differentiate to cell derivates of the three germ layers. This also corresponds to the level of development of the stem cell itself.

Embryonic stem cells are pluripotent cells that can differentiate to cells representing all three germ layers. Mesenchymal stem cells are only able to differentiate into cells derived from the mesoderm layer. Stem cells of both embryonic and mesenchymal origin have the potential to differentiate into the main tissue components found in the VFs.

Animal experiments are critical for studying the pathogenesis, prevention and treatment of VF scarring (Rousseau et al., 2003). There are no other ways to conduct systematic research on the biomechanical properties of the complicated layered structure of the VF, lamina propria. Animal model studies are essential for the development of prevention and treatment strategies for the complex clinical problem of scar formation in the VFs (Bless et al., 2010).

1.1 DEVELOPMENT OF THE VOCAL FOLDS

In the newborns the vocal folds (VFs) are immature. Sato and Hirano found that they have no vocal ligament. The Lp is a uniform monolayered structure not yet stratified. This monolayer is a loose viscous structure rich in hyaluronic acid, a glycosaminoglycan with strong affinity to water. The monolayed Lp also contains ground substances such as elastin, fibronectin, fibroblasts, and collagen fibers. The

reticular fibers, elastin, are few. Fibronectin is abundant. Fibronectin is a glycoprotein that acts as a stabilizer for the elastic tissue formation and the collagen fibrils. The fibroblasts are oval shaped, and has a large nucleus-cytoplasm ratio, indicating inactivity. The collagen fibers are few and are not diversified in structured layers.

By growth into infancy the newborn VFs orientate the elastic and collagen fibers longitudinally in the entire lamina propria (Sato et al., 2001). Fibronectin is believed to guide the orientation of the collagen fibers. The fibroblasts are still sparse but become more spindle or stellate shaped.

Sato found that by the age of 2 months a separation into a bilamilar Lp could be noticed with a superficial layer being less densely populated than the deeper layer. By 11 months, a tendency to a three-layered Lp can be seen with a superficial hypocellular layer, an intermediate some hypercellular layer, and a deeper thin more hypercellular layer. By 7 years of age the middle layer is still some hypercellular, but also a greater content of elastin and collagen fibers can be seen. The deeper layer starts by then to be less cellularly populated.

At age13 the hypocellular superficial layer persists. The intermediate layer now consists of predominantly elastin fibers, and the deeper layer mostly of collagen fibers. This maturation seems to be ongoing to the age of 17, when the intermediate layer of elastic fibers and the deeper layer of collagen fiber structures seem to have matured (Sato et al., 2001; Hartnick et al., 2005).

The content of the ground substances seems to decrease over time, as the fibrous components increase, gradually changing the vocal fold structure into an adult VF. Which mechanisms though, that regulates this maturation process is still unknown. However, there are indications that vibratory stimulation induces maturing biological changes in the VFs. Sato et al analyzed the VFs of adults that had had no own speech and found that the VFs had no vocal ligament and the Lp lacked its three layered structure. Instead the Lp showed a mono structure more like the VFs of newborns. In the maculae flavae the stellate cells showed signs of inactivity and degeneration (Sato et al., 2008).

Titze showed that fibroblasts that are exposed to mechanical stimulation produce different levels of extra cellular matrix proteins than fibroblasts that are not exposed to mechanical stimulation (Titze et al., 2004). The gene expression levels of the extra cellular matrix components such as fibronectin, decorin, fibromodulin, matrix metalloproteinase-1 (MMP1), HA synthase 2 and CD44 were found altered. These genes are all involved in the extra cellular matrix remodeling by age. This indicates that mechanical forces applied to the tissue, alter the expression levels of extra cellular matrix related genes.

Hormones influence the voice alteration in puberty. How this is done though is poorly investigated. The presence of receptors for androgen, progesterone and estrogen in several cells in the VFs, suggests that some of the developmental changes and structural differences seen by growth and gender are likely to be caused by hormonal influences (Newman et al., 2000). As expected more androgen but also progesterone receptors have been found in male compared with female VFs (Hammond et al., 1998). Thus it is

suggested that the estrogen/androgen ratio is responsible for the voice changes observed at menopause (Nelson, 1995).

1.2 ADULT VOCAL FOLDS

The adult human VF is covered by a stratified squamous epithelium. The VF has a clearly defined three layered structured lamina propria (Lp) (Hirano et al., 1981; Gray et al., 2000; Hartnick et al., 2005). The superficial layer (sLp), the intermediate layer (iLp), and the deep layer (dLp). The different layers are distinguished by the content of the extra cellular molecules and by the content of elastic and fibrous elements, which are elastin and collagen compositions. The sLp, has fewer elastic and collagenous fibers than the two deeper layers. This makes the sLp looser and extremely pliable (Hirano and Kakita, 1985; Gunter, 2003).

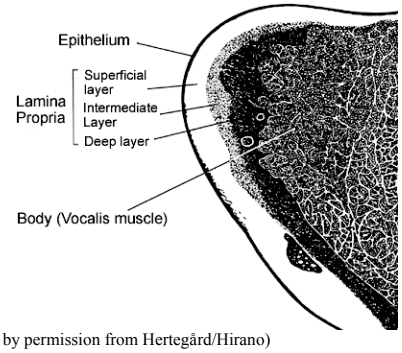

Figure 1: Adult vocal fold (Adapted by permission from Hertegård/Hirano)

The intermediate layer is mostly composed of elastic fibers, while the deeper layer is dominated by collagenous fibers with less elastic fibers (Hammond et al., 1997). The intermediate and deeper layers form the vocal ligament. The collagen fibers are organized in longitudinal bundles along the entire vocal ligament (Hirano et al., 1981).
Elastic and collagenous fibers play different roles in the Lp. Elastin fibers bring elasticity to the tissue, which allows it to return to its original shape after deformation. The content of elastin in the Lp is over twice of that found in dermis (Hahn et al., 2006). Collagen, mostly type I render strength to the tissue, in order to withstand stress and resist deformation when subjected to a force (Gray, 2000).

The structure of the intermediate and deeper layers of the Lp, i.e. the vocal ligament balances the tension from the vocal muscle with that from the superficial oscillating layer of the Lp. (Hirano and Kakita, 1985; Rosen, 2000).

The extracellular matrix (ECM) of the Lp also contains interstitial proteins such as hyaluronic acid (HA), a non-sulfated glycosaminoglycan. With its strong affinity to water HA is essential for the tissue viscosity. HA also acts as space-filler and shock absorber (Chan et al., 2001; Schweinfurth et al., 2008), as well as wound healing stimulator promoting cell migration (Ward et al., 2002).

Under the vocal ligament is the vocal muscle i.e. the thyroarytenoid muscle. The thyroarytenoid muscle is separated in a superficial and a deeper part.

The superficial layer of the Lp is also called the Reinke's space (Hirano, 1981; Dräger et al., 2011). At times the definition "the mucosa" of the VF is used, which includes the epithelium and the space of Reinke.

1.3 MACULAE FLAVAE

There are two dense parts, one at each end, of the membranous VF, named macula flava.

It has newly been shown that newborns have immature maculae flavae at the same sites as adults (Sato et al., (part 2), 2010). The maculae flavae both in newborns and in adults have been shown to be composed of dense masses of vocal fold stellate shaped fibroblast like cells (VFSCs). These cells might be of an entity of their own in the VFs (Sato and Hirano, 1995). These cells have intracellular organelles and a number of vesicles are constantly present at the periphery of the cytoplasm of the cells indicating a flow of synthesizing activity (Sato et al., (part 1), 2010).

Sato and Hirano have suggested that the maculae flavae are hosting the production of the cell components and proteins of the Lp. It has been speculated that activated stellate shaped fibroblasts in the maculae flavae are the production source (Sato and Hirano, 1995; Sato et al., (part 1), 2010).

Interestingly, stellate cells are also found in the liver representing about 5% of the total number of liver cells (Geerts et al., 2001). Each cell has several long protrusions that extend from the cell body and wrap around the sinusoids. They also contain lipid droplets that store vitamin A, just as the stellate cells in the VFs (Stanciu et al., 2002; Sato et al., 2008).

The hepatic stellate cells have been held responsible for secreting collagen scar tissue, causing cirrhosis, but also to limit liver fibrosis by activating the immune system by interacting with natural killer cells (Krizhanovsky et al., 2008).

As mentioned, vitamin A has also been found in the stellate shaped fibroblasts of the maculae flavae in the VFs of rats. Vitamin A has been shown to be important in the development of the larynx in rats (Tateya et al., 2007). It has also shown to be important for the maintenance of the vocal fold epithelium cells in rats (Tateya et al., 2008).

Less scarring has been observed with increased amount of vitamin A in injured human VFs (Akdogan et al., 2009).

1.4 RABBIT VOCAL FOLDS

1.4.1 Anatomy of rabbit vocal folds

The rabbit VF is covered by a similar stratified squamous epithelium as human VFs. The rabbit VF has a well-defined Lp. However, the three layered structure of a superficial, an intermediate and a deep layer seen in the human Lp is not clearly found in the VFs of rabbits. The rabbit Lp is divided into two layers, a superior and a deeper layer (Kurita et al., 1983). The deeper layer of the rabbit VF is the structure that corresponds to the vocal

ligament of the human VF. Under the two layered Lp in the rabbit VF is the vocal muscle. The thyroarytenoid muscle is separated in a superficial and a deeper part as in human VFs.

The well-defined Lp of the rabbit VF is built up by a scaffold structure as the Lp in the human VF. This scaffold or extra cellular matrix, consists of fibers of elastin and collagen as in the human Lp (Branski et al., 2005; Hirano et al., 2009). The most common extra cellular components in this matrix are constituents of the same proteins, proteoglycans, glucosaminoglukans, fibroblasts and inflammatory cells that are found in the VF of humans (Thibeault et al., 2003; Hirano et al., 2009).

1.4.2 Viscoelasticity in the rabbit vocal fold

The viscoelastic shear properties of normal rabbit VF tissues are found be similar to those of normal human VF tissues (Thibeault, 2002).

1.5 COMPLEXITY OF SCARRING

1.5.1 Dermal scarring

Scarring after dermal injuries is well investigated. The damage of tissue and blood vessels initiates the inflammatory response by releasing chemo attractants, cytokines, and growth factors into the wound. These recruit macrophages, neutrophils and monocytes to the site and this stimulates the keratinocytes and fibroblasts to initiate the repair process (Hackam and Ford, 2002).

Macrophages release numerous of cytokines that act as keys to fibroblast chemotaxis and proliferation. These cytokines include platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-beta (TGF-β).

In addition, macrophage-derived cytokines such as interleukin-4 (IL-4) stimulate the formation of granulation tissue i.e., collagen production (Clark, 1996).

During the first 2 days after injury the keratinocytes migrate into the wound site (O'Toole, 2001). The keratinocytes at the periphery are stimulated by the epidermal growth factor and the fibroblast growth factor (FGF), released from both the inflammatory cells and tissue fibroblasts (Werner, 1998; Lawrence, 1998; Martin, 1997). The re-establishment of an epithelium is a key aspect. A dermal wound is re-epithelialized when a water-impermeable seal is present over the wound (Hackam and Ford, 2002).

Wound contraction starts at day 4 to 5 after injury (Lawrence, 1998). Contraction is characterized by a centripetal movement of the wound edge toward the center of the wound. Both fibroblasts and myofibroblasts are thought to be responsible for this process (McGrath and Hundahl, 1982; Ehrlich, 2000).

Scar remodeling is the final stage of wound healing. The amount of collagen becomes stable at approximately 3 weeks after injury (Lawrence, 1998; Martins-Green, 1997).

However, the remodeling involving synthesis and degradation of collagen continues thereafter for several months, and it contributes to the gradual regain of the tensile strength of the tissue.

1.5.2 Vocal Fold scarring

In injured rabbit VFs a massive cellular infiltration of inflammatory cells and fibroblasts is seen on day 2-3 (Branski et al., 2005; Campagnolo et al., 2010).

Hyaluronic acid, collagen and fibronectin deposition are most prominent at day 3-5 in rats (Tateya et al., 2006).

In rabbits a complete hypertrophic epithelial coverage is seen on day 5. On day 5 a neo-collagen matrix is also noted (Branski et al., 2005).

In rabbits interleukin I (IL-1) returned to baseline levels by day 7 after injury. Prostaglandin E 2 (PGE-2) concentrations remained elevated passed day 21, with maximum expression on day 7 post injury (Ryan et al., 2005).

"More" mature collagen is seen by day 7. Dense unorganized collagen is noted by day10. Little change takes place from day 10 to 14. By day 21 the neo-matrix appears laminated, with significantly increased density of fibrous tissue (Branski et al., 2005).

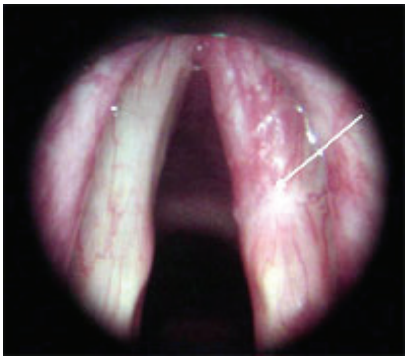

Figure 2: Human vocal fold scar

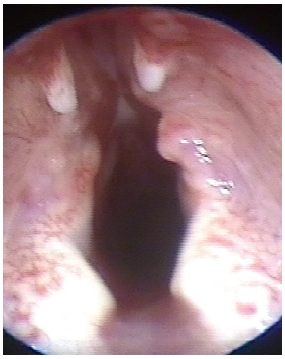

Figure 3: Rabbit vocal fold scar

1.5.3 Vocal Fold scar

Scaring in the Lp of the VF is due to a destruction of the delicate network built up by the fibrous proteins, elastin and collagen and altered concentrations of the interstitial protein molecules within this network. The fine network of elastin and collagen fibers is in a VF scar replaced by abundant disorganized bundles of collagen (Benninger et al., 1996). Disarranged collagen architecture in the vocal fold has been found to be associated with increased viscosity and stiffness of the VF (Thibeault et al., 2002).

Interstitial proteins are molecules that consist of protein chains connected to polysaccharide branches. They are known to play an important role in the cell interactions

(Tibeault et al., 2003). Three interstitial protein molecules have been found to be of special interest.

1.5.3.1 Decorin

Decorin, also named proteoglycan II (PGII), is a leucine rich glucosaminoglukan with a sulphate chain. The sulphate chain has been shown to attach to collagen type I modifying collagen fibril formation (Krusius and Ruoslahti, 1986).

In rabbits decorin is distributed throughout the layers of the VF both in scarred and normal VFs (Thibeault et al., 2003). Scarred vocal folds have lower density of decorin than normal VFs (Scott et al., 1998). Reduced decorin causes disturbed collagen fiber growth and renders disorganized collagen fibers. Disorganized collagen fibers are described in acute VF scar formation (Thibeault et al., 2002).

1.5.3.2 Fibromodulin

Fibromodulin is also a small leucine-rich proteoglycan. It belongs to the same group of smaller interstitial proteoglycans as decorin. The core proteins of these proteoglycans are structurally related, consisting of a central region of leucine-rich repeats with attached sulfate chains. Fibromodulin like decorin thus binds to collagen (Hedbom and Heinegard, 1989).

In rabbits fibromodulin have been found to be decreased in scarred vocal fold tissue compared to normal VFs (Thibeault et al., 2003). Reduced fibromodulin has also been observed in dermal scaring (Soo et al., 2000).

Fibromodulin inhibits the transforming growth factor-β (TGF-β). TGF-β upregulates the synthesis of collagen (Frenzel, 1986; Ignotz, 1986). Thus a reduced amount of fibromodulin increases the collagen production.

Increased levels of the collagen precursor procollagen I have been found in acute scars of VFs (Thibeault et al., 2002). The presence of procollagen points to an ongoing production of new collagen fibers in acute scar formation. New production of collagen fibers stimulated by reduced levels of fibromodulin with concomitantly reduced levels of organizing decorin gives some explanation to the finding of increased production of disorganized collagen bundles seen in VF scars.

Hepatocyte growth factor (HGF) and its receptor c-Met are shown to be present on injured rabbit vocal fold gland cells. Prominent HGF activity in injured rabbit VF Lp and epithelium is seen on days 10 and 15 after injury (Hirano et al., 2002).

Hirano have shown that HGF in human VFs upregulates the production of HA by Reinke space fibroblasts (FbRS), and downregulates the fibroblasts production of collagen type I by the FbRS and fibroblasts in maculae flavae (FbMF) (Hirano et al., 2003).

1.5.3.3 Fibronectin

Fibronectin is a signaling protein necessary for wound healing. It produces adhesion facilities necessary for the attachment of epidermal cells to their basement membrane. Fibronectin also stimulates cell migration and replication (Ongenae et al., 2006).

Fibronectin is found mainly in the superficial layer of the Lp both in normal and scarred VFs. It is scarcely seen in deeper layers. It has been shown to be increased in scarred VFs in comparison with normal VFs (Thibeault et al., 2003). A rise in fibronectin concentration in VF scar formations is in accordance with observations done in dermatology studies of cutaneous scars (Kischer and Hendrix, 1983; Ongenae, 2000).

Fibronectin does not bind to collagen. The transforming growth factor-β (TGF-β) stimulates the production of fibronectin (Ignotz, 1986).

Thus a reduction in fibromodulin with lowered inhibition of the transforming growth factor-β (TGF-β) will increase the level of fibronectin and tissue production. A concomitant reduction in decorin will simultaneously increase collagen production. In addition the transforming growth factor-β (TGF-β) has a direct stimulation on collagen growth.

There might be a window of appropriate inter levels of the extra cellular proteins of decorin, fibromodulin and fibronectin that is a necessity to maintain for an optimal stimulation of renewal activity. An increased activity can be expected to form abundant disorganized scar formations. The transforming growth factor-β (TGF-β) seems to play a central role as an interregulator, with its direct ability to stimulate collagen synthesis, to down regulate the production of decorin and to increase the production of fibronectin. But it is itself down regulated by an increase in fibromodulin. Hence the fibromodulin may play the key role in this equilibrium (Hildebrand et al., 1994). However several other protein components intermix. The full story is not yet revealed in this intricate extracellular concurrence.

1.5.4 Hyaluronic acid

Hyaluronan is a glycosaminoglycan, a polymer of disaccharides. Hyaluronan is synthesized by membrane proteins called hyaluronan synthases. Vertebrates have three types: HAS1, HAS2, and HAS3. These enzymes lengthen hyaluronan by repeatedly adding glucuronic acid and N-acetylglucosamine to the polysaccharide chain as it is extruded through the cell membrane into the extracellular space (Schulz et al., 2007).

The hyaluronan is predominantly found in the deeper part of the Lp of the VF.
It is essential for the viscosity as it richly binds water molecules. Thus it also serves mechanically as shock absorbent (Chan et al., 2001).

In the early inflammatory phase of skin wound repair HA is abundant as a reflection of increased synthesis. It has been shown that HA acts as a promoter of early inflammation and is crucial in the skin wound-healing process (Chen and Abatangelo, 1999). HA enhances cellular infiltration (Wisniewski, 1996). The cell infiltration is essential for the

formation of granulation tissue. The early stage of granulation tissue is dominated by a HA-rich extracellular matrix (Chen and Abatangelo, 1999).

HA provides an open hydrated matrix that facilitates cell migration (Ellis et al., 1996). The migration control of the cells, the locomotory mechanism, is mediated by specific cell surface HA receptors. The three principal cell surface receptors for HA are the CD44, receptor for hyaluronic acid mediated motility (RHAMM), and inter-cellular adhesion molecule 1 (ICAM-1). RHAMM is believed to be specifically related to cell migration (Hall et al., 1996; Nedvetzki et al., 2004). Cell movement can be inhibited and growth of rat embryos stagnate, at least partially, by HA reduction or by blocking HA receptors (Morriss-Kay et al., 1986).

A dose-dependent increase of the pro inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and IL-8 production by a CD44-mediated mechanism has been showed in uterine fibroblasts of humans (Kobayashi and Terao, 1997). The cytokin TNF-α has been found to stimulate the expression of TSG-6 (TNF-stimulated gene 6) in fibroblasts and inflammatory cells. TSG-6, is a HA-binding protein and forms a stable complex with the serum proteinase inhibitor IαI (Inter-α-inhibitor) which has a synergistic effect on the plasmin-inhibitory activity. Plasmin activates the proteolytic cascade of proteinases causing inflammatory damage on the granulation tissue. The TSG-6/ IαI complex may serve as a potent negative feedback loop to moderate inflammation and stabilize the granulation tissue in the healing progresses (Chen and Abatangelo, 1999; Wisniewski et al., 1996)

The HA content of the human Lp is similar to that of the dermis (Hahn et al., 2006).

In injured VFs of rabbits HA has been found to be under the levels of normal VFs to day 15 after injury except on day 5 where equal levels were found (Thibeault et al., 2004, 2005).

In rabbit VFs the hyaluronan levels at 2 and 6 months post injury are found equal between scarred and normal VFs (Thibeault et al., 2002; Rousseau et al., 2004).

A study of 6 months duration with injection of hyaluronic acid hydrogel into injured VFs of rabbits showed a down regulation of fibronectin, fibromodulin, procollagen I, hyaluronic acid synthase and transforming growth factor-β1 (TGF-β1) transcription compared with untreated controls (Thibeault et al., 2011).

Being an essential player in the structure of the extra cellular matrix the reduction of HA content in the early phase of wound repair but normalized by 2 and also 6 months post injury, together with the improved rheological parameters after addition of HA at time of injury (Thibeault et al., 2011), suggests that the critical time period of wound repair in the VFs might be the first few days or weeks after injury.

1.5.5 Collagen

Collagen is a group of proteins richly found in connective tissues of animals. Approximately 34 genes are known to be associated with collagen formation. Collagen is

composed of a triple helix synthesized within the cell of mostly fibroblasts. The triple helix is known as procollagen. It consists of two identical chains and an additional chain that differs in the amino acid sequences (Traub et al., 1969).

When secreted out of the cell, cell membrane bound enzymes proteolytically remove no longer needed signaling end peptides, carboxyl and amino terminal propeptides, from the procollagen molecule. What is left is known as tropocollagen (Prockop et al., 1979; McDonald et al., 1986).

In the extracellular matrix lysyl oxidase induces bonding between tropocollagen molecules forming a polymer, a collagen fibril. Bundles of collagen fibrils form collagen fibers (Hulmes and Miller, 1979; Jesior et al., 1980).

More than 27 different types of collage have been described (Myllyharju, and Kivirikko, 2004). The different types can be classified and measured after which end propeptides that are removed when secreted from the producing fibroblast.

The most common collagen types are collagen type I-III. Collagen type I is the most abundant collagen in the human body. It is found in tendons, skin, and bones and in scar tissue. Collagen type I forms about 90% of the collagen found in humans (Lodish et al., 1999).

Collagen type II makes up most of the collagen in cartilage. Collagen type III is found in abundance in granulation tissue. It is the prime collagen type produced by the fibroblasts in dermal scars before the stronger type I collagen is synthesized (Prockop et al., 1979; Martinez et al., 1994).

1.5.5.1 Collagen in human vocal folds

Collagen type III is found throughout the human Lp with the largest concentration in the deeper layer (Bühler et al., 2011). Collagen type I is localized mainly just beneath the basal membrane and in the deep layer of the Lp. It is also found in the anterior and posterior maculae flavae (Tateya et al., 2006).

Both collagen types I and III are found in the intermediate layer though predominantly collagen type III (Madruga et al., 2003; Prades et al., 2010).

In the VFs collagen type III has been described as having a more wavy form compared with type I having thinner more compact fibers (Tateya et al., 2006).

Collagen type III may be the primary type of collagen in the human adult Lp (Tateya et al., 2007). It may compose 40% of the total Lp collagen content (Hahn et al., 2006). The content of collagen type III in the vocal fold Lp is in parity with highly elastic lung parenchyma, and twice of the amount found in dermis (van Kuppevelt et al., 1995; Epstein et al., 1978).

Interestingly the collagen type III has been found to be less than 10% of the total collagen content in the childhood Lp (Muñoz-Pinto et al., 2009).

In addition to the type of collagen, collagen fiber thickness and orientation of the collagen fibers may contribute to the viscoelastic properties of the VF. It has been shown that the average thickness of the collagen fibers increases with increasing Lp depth, with the thinnest collagen fibers in the superficial layer and the thickest fibers in the deeper layer (Muñoz-Pinto et al., 2009).

1.5.5.2 Collagen in rabbit vocal folds

In normal rabbit VFs procollagen I is found almost exclusively in the superficial Lp. The density increases by scarring but returns to normal level at 2 months post injury and are found to stay so at 6 months (Thibeault, 2002, Rousseau et al., 2004)

In scarred VFs collagen is distributed throughout the Lp. Scarring shows at 2 months non organized, less dense collagen (Thibeault, 2002).

A remodeling phase of collagen in the scar tissue starts at 3 weeks post injury and may continue to 6 months. Maturing of elastin may continue for a year (Rousseau et al., 2004).

At 6 months the collagen is found to be increased, organized, and to have formed thick bundles in scarred VFs. For comparison, elastin that in healthy VFs is mostly found in the deeper layers is found all through the Lp at six months. It is decreased by 2 months and is still decreased and disorganized at six months post operatively (Rousseau et al., 2003, 2004).

1.6 STEM CELLS

Stem cells are immature cells. They are the cell type of origin to all cells in the body. They divide and differentiate to specialized cells in the growing process of the embryo. They have the potential to divide into identical cells as themselves or choose to differentiate to more specialized cells. The ability to divide into identical copies of themselves is called self-renewal. Self-renewal means that the stem cells can divide while maintaining their undifferentiated state.

The stem cells are also characterized by their ability to which cells in the body they can develop into. This ability refers to the stem cell`s own development. Their development gradually reduces their ability to differentiate into the variety of organ specific cells. Hence due to the developmental stage of the stem cell, it is characterized after the types of specialized cells that it can differentiate into. Pluripotent stem cells can differentiate into all cells in the body of the animal or human from which they originates.

Multipotent stem cells can develop into a numerous of specialized cell types but not all. That means that they can only produce a limited variety of specialized cells. The limitation corresponds to the development of the stem cell into one of the three primary germ layers, ectoderm, endoderm or mesoderm.

Thus stem cells can also be classified after their own developmental state. Embryonic stem cells (ESCs) are stem cells in the developmental stage before the differentiation in to

the three germ layers takes place. Thus ESCs have the potential to develop into cells of all the three germ layers.

Mesenchymal stem cells are stem cells that have developed into the mesoderm stage and have the potential to develop into specialized cells of the mesoderm germ layer. Mesenchymal stem cells are defined as multipotent. That means that they can differentiate to two or more specialized cell types.

Stem cells can also be classified as embryonic or adult stem cells. There are a variety of adult stem cells. Adult stem cells are found among differentiated cells in the tissue of organs and can differentiate into the specialized cell types found in the tissue of that organ. Adult stem cells are found in several tissues in a rare or less abundant amount. A source to stem cells newly shown is induced pluripotent stem cells. These cells are through transcription factors, genetically reprogrammed mature adult cells. These reprogramed cells are known as induced pluripotent stem cells, iPS-cells, with the characteristics of pluripotency like ESCs (Yu J et al., 2007; Okita and Yamanaka, 2010).

In paper II human embryonic stem cells were used. Human mesenchymal stem cells were used in Paper I and III-V.

1.6.1 Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst, prior to implantation. For human ESCs that is day 5-6 post fertilization. At this time all the cells in the inner cell mass have the potential to develop into any cell of the three germ layers. Thus the ESC can differentiate into any cell in the body. The ESC also has the capacity of self-renewal. This gives each ESC theoretically unlimited proliferative potential. The maintenance of the immaturity, pluripotency and the self-renewal capacity of the ESCs are strictly regulated both on the transcriptional and on the cell cycle level.

1.6.2 Human Embryonic stem cells

The regulation of human embryonic stem cells (hESCs) on the transcriptional level is mainly directed by the three transcription factors Oct 4-Sox2-Nanog. These three transcription factors inhibit differentiation of hESCs and keep them in the pluripotency state. (Nichols et al., 1998; Avilion et al., 2003; Mitsui et al., 2003; Johnsen, et al., 2004)

In the cell cycle the regulation of the gene p53 and the Rb-pathway inhibit cell cycle progression after DNA damage, ensuring either apoptosis or cell repair. P53 is also active in cell differentiation, migration and programmed cell death (Lane, 1992).

These systems serve as a protection in the somatic cells from tumor genesis. The 53p and Rb are the two most important tumor suppressors. Rb stands for Retinoblastoma protein. It was first discovered being the gene most often mutated in retroblastomas. Both the 53p and the Rb-pathways are strongly associated with tumor genesis as all tumors have a defect in at least one of these genes. Fifty percent of all tumors have a mutated 53p gene

(Hainaut and Hollstein, 2000; Hong et al., 2009; Vurusaner et al., 2011).

In cell cultures the hESCs lack or show low expression of gen p53 and with p53 collaborating genes (Brandenberger et al., 2004). Also genes in the Rb-pathway are found to be down regulated (Miura et al., 2004). Therefore it is essential to control the genotype of the hESCs regularly and prior to implantation.

The telomeras activity in hESCs has been found to be elevated (Thomson et al., 1998; Carpenter et al., 2004).

Furthermore, it has also been shown that epigenetic proteins contribute to inhibit differentiation of the hESCs and thus assist in maintaining them in pluripotency (Schuettengruber et al., 2007).

All these characteristics in the hESCs are elements that reveal the property of unlimited self-renewal of hESCs.

1.6.3 Human Embryonic stem cell line HS 181

The first human ESC line was cultured by Thomson in 1998 (Thomson et al., 1998).

The ESCs were cultured on a layer of murine fibroblasts. The fibroblasts were used as feeder cells providing a matrix to favor the growth of the ESCs.

In 2003 the stem cell line HS 181 was derived by the hESC-network at the Karolinska Institutet (Hovatta et al., 2003). The cell line is of karyotype 46[xx] i.e. of female genotype.

This stem cell line was kindly provided by Professor Outi Hovatta and was used in the study described in paper II.

The HS 181 cells were grown on mitotically inactivated human foreskin fibroblasts as feeder cells. The technique is described by Imreh MP et al., 2004. The feeder cells were mitotically inactivated using irradiation with 35 Gy before seeding. Embryonic stem cells in culture require basic fibroblast growth factor (bFGF or FGF-2) for growth. Without optimal culture conditions, embryonic stem cells in culture will rapidly differentiate (Chambers et al., 2003).

HS181 cells corresponding to passage 32 from two separate thawings were used in the study described in paper II. These cells were kindly provided by Professor L. Ährlund-Richter.

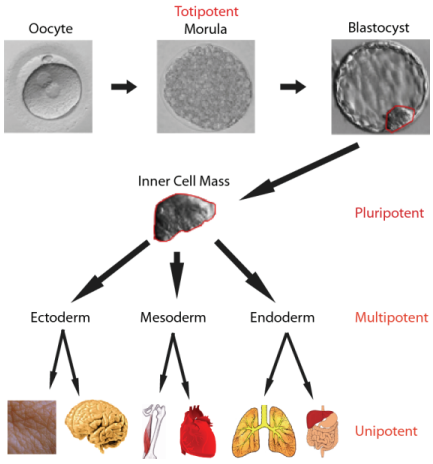

Figure 4: Embryonic stem cell development

1.6.4 Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) were first identified by Friedenstein et al., in 1970**.** They observed a group of cells that developed into fibroblastic like colonies. These cells were referred to as colony-forming unit-fibroblasts (CFU-F).

These cells later became known as bone marrow derived stromal cells. They were found to be multipotent and to host the capacity of self-renewal (Nauta et al., 2007). These bone marrow derived stromal cells now known as mesenchymal stem cells (MSCs) are multipotent self-renewing progenitor cells of mesenchymal tissues. The multipotency refers to the ability of the MSCs to differentiate to several but not all cells in the body. This limitation differentiates them from embryonic stem cells. Thus MSCs have the potential to differentiate into connective tissue cells, muscle, cartilage and bone that is, myocytes, chondrocytes, osteoblasts and adipocytes, as well as to pneumocytes, hepatocytes and cardiomyocytes.

MSCs represent 0,001-001% of the nucleus cells in the bone marrow (Wu Yet al., 2007). Human MSCs are expandable in cell cultures (Pittenger et al., 1999; Orlic et al., 2001).

Human MSCs have been shown to have the capacity to leave the bone marrow, and circulate in the blood and home into injured tissues (Fernandez et al., 1997; Asahara et al., 1999)

When human infants with osteogenesis imperfecta received autologous MSCs administered systemically the MSCs were found to home to bone where the injected MSCs participated in de novo osteogenesis. They were found to form new dense bone and to increase the total bone mineral content (Horwitz et al., 1999).

It has been shown that when an autologous intravenous infusion of MSCs is administrated to rats with induced stroke the MSCs reduce the size of the scar in the brain with reduced number of gliacells and enhance axonal growth in the lesion (Li Y et al., 2005).

In a mice model with bleomycin-treated mice, systemically injected MSCs were found to home to the injured lungs and adopt an epithelium-like phenotype. The MSCs were also found to significantly reduce the bleomycin-induced inflammation and the collagen deposition in the lungs (Ortiz et al., 2003).

Human MSCs are found to be immunosuppressive and to reduce lymphocyte proliferation and formation of cytotoxic T-cells (Le Blanc et al., 2003). MSCs are also found to enhance secretion of anti-inflammatory cytokines and chemokines thus shifting from pro inflammatory to anti inflammatory cytokines at the site of injury (Ryan et al., 2005; Nauta et al., 2007; Chen et al., 2008).

Le Blanc have shown that a severe steroid-resistant acute graft-versus-host reaction after gut and liver transplantation in a young individual could be reversed by infusion of haploidentical MSCs (Le Blanc et al., 2004).

Vascular delivery of stem cells suffers from a "pulmonary first pass effect" where intravenously injected cells are partly sequestered in the lungs (Fischer et al., 2009). Therefore efforts have been done to promote mobilizing of the bone marrow to deliver their MSCs into the blood stream in order to let the freed MSCs target to the site of injury, i.e. a cutaneous wound, the myocardial infarct area or the stroke area in the brain, or in principle at any wound site (Lambert et al., 2009).

A number of hematopoeitic cytokines have been found to mobilize bone marrow stored MSCs into the blood stream, such as granulocyte colony stimulating factor (G-CSF) (filgrastism), granulocyte macrophage colony stimulating factor (GM-CSF) (sargramostim). Thrombopoieten (TPO), FMS-like tyrosine kinase 3 ligand (FLT-3L) and interleukin-8 (IL8) have also exhibit effects in mobilizing human MSCs to peripheral blood from the bone marrow. Although this form of stimulating the release of MSCs from the bone marrow increases the total amount of MSCs, the MSCs at the target site will still be relatively few. Therefore it has been proposed that direct injection or placement of MSCs into the site in need of repair may be the preferred method of treatment (Fischer et al., 2009; Wu Y et al., 2007).

Premobilization of MSCs into peripheral blood by granulocyte colony stimulating factor (G-CSF) before an induced acute myocardial infarction in mice, resulted in homing and engraftment of MSCs in the myocardial infarct area. A significant degree of myocardial transdifferentiated regeneration was believed to be noted (Orlic et al., 2001).

However, transdifferentiation of MSCs into myocytes and into other cells in the myocardium have not been observed by other researchers. Although these researchers also found regeneration of cardiomyocytes with improved healing this was in these studies found to be due to cell fusion (Nygren et al., 2004). The regeneration of cardiomyocytes by fusion suggests that the MSCs mediated the myocardial repair or regeneration not by transdifferentiation but through paracrine signalling or other mechanisms (Balsam et al., 2004).

The finding that cultured bone marrow derived MSCs have been found to release vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-6, placental growth factor (PlGF), and monocyte chemo attractant protein-1, support the assumption that a substantial paracrine influence can be expected from bone marrow derived MSCs (Kinnaird et al., 2004).

1.6.5 Mesenchymal stem cell preparations – Culturing

To culture human MSCs (hMSCs) original unpurified bone marrow (BM) or purified BM can be used. The BM is plated directly into cell culture plates or flasks. MSCs adhere to tissue culture plastic within 24 to 48 hours. The red blood cells and hematopoietic progenitors are not adherent to plastic. The MSCs can thus be rather easily separated.

The hMSCs used in the studies described in paper I, III-V, were isolated and expanded from bone BM taken from the iliac crest of healthy volunteers. The cells were selected on the basis of optimal cell growth and differentiation. The cells were harvested averagely in passage five. Detailed culture conditions are stated in *MATERIAL AND METHODS*.

1.6.6 Mesenchymal stem cell characterization (Identification of Mesenchymal Stem Cells)

The most commonly reported positive markers for hMSCs are CD13, CD29, CD34, CD44, CD54 CD73, CD90, CD105, CD106, CD146 and CD166. The most frequently reported negative markers are CD10, CD11b, CD14, CD31, CD34, CD45, CD49d and CD106. A number of other cell surface markers have also been reported, such as STRO-1, SH2, SH3, SH4, HLA-A, HLA-B, HLA-C, HLA-DR, HLA-I, DP, EMA, DQ (MHC Class II), CDIO5, Oct 4, Oct 4A, Nanog, Sox-2, TERT, Stat-3, fibroblast surface antigen, smooth muscle alpha-actin, vimentin, integrin subunits alpha4, alpha5, beta1, integrins alphavbeta3 and alphavbeta5 and ICAM-1 (Mafi et al., 2011).

The hMSCs used in the studies described in paper I and III-V, were identified as mesenchymal stem cells based on their ability to differentiate into adipocytes, chondrocytes and osteocytes when cultured in adipogenic, chondrogenic or osteogenic media. They were classified by flow cytometric analyses. They were found positive to CD73, CD90, CD105 and CD166 and negative to CD14, CD31, CD34, CD45 and CD80. Detailed conditions for the classification are stated in *MATERIAL AND METHODS*.

2 OBJECTIVES

The overall aim of this thesis was to explore if human stem cells transplanted in a scarred vocal fold (VF) have the potential to restore the histological and rheological properties of the VF.

Using a xenograft model with rabbits the following aims were initially set;

- To explore if human stem cells can graft in rabbit vocal folds.
- To explore if human stem cells stimulate the healing process in scarred VFs.
- To explore if human stem cells can regenerate VF tissue.
- To explore if stem cell promoted healing or tissue restoration can be expected to be persistent.
- To explore eventual effect differences between transplanted human embryonic and human mesenchymal stem cells on scarred VFs.
- To find a model for treatment of scarred VFs using human stem cells.

3 MATERIAL AND METHODS

3.1 STEM CELLS

3.1.1 Human embryonic stem cells

Human embryonic stem cells (hESCs) of the cell line HS181 (Hovatta et al., 2003) were used and studied in paper II.

The hESC line HS181 passage 32 was kindly provided by Professor L. Ährlund-Richter, Karolinska Institutet.

3.1.2 Culture conditions for human embryonic stem cells

The HS181 cells were cultured as described by Imreh et al 2004; i.e. maintained in 80% KnockOut-Dulbecco's Modified Eagle Medium (KO-DMEM), 20% KnockOut-Serum Replacement (KO-SR), 2 mM L-glutamine, 1% nonessential amino acids, 0.1 mM beta-mercapoethanol, 4 ng/ml basic fibroblast growth factor (bFGF); at 37°C, 6.8% CO2 and high humidity. Commercially available human foreskin fibroblasts (CRL-2429; ATCC, Manassas) were used as feeder cells. The fibroblasts were cultured in IMDM, supplemented with 10% fetal bovine serum (FBS). The feeder cells were mitotically inactivated by irradiation at 35Gy before seeding on a 6-well plate (Corning, NY) at a density of $2.1x10^4$ cells/cm^2.

The HESC culture medium was changed daily and the cells were passaged every 4–6 days by incubating in dispase enzyme (10 mg/ml) for 5 – 7 min at 37°C and mild mechanical splitting. (All reagents obtained from Invitrogen AB, Stockholm, Sweden).

Passage 32 was used in this study. Before, during, and after this study, the cells were classified as hESCs by their functional pluripotency *in vivo* (teratoma assay; see Gertow et al 2007), a normal karyotype (46XX), as well being shown to be positive for, Oct-4, SSEA-4, Tra1-60, Tra1-81, and alkaline phosphatase, while being negative for SSEA-1 (performed as described by Imreh et al., 2004).

3.2 HUMAN MESENCHYMAL STEM CELLS

Bone marrow derived human mesenchymal stem cells (hMSCs) were used and studied in paper I and III-V.

The hMSCs were kindly provided by Professor K. Le Blanc Karolinska Institutet.

3.2.1 Culture conditions for human mesenchymal stem cells

The hMSCs were isolated and expanded from bone marrow (BM), taken from the iliac crest of healthy volunteers. The hMSCs were cultured as described by Le Blanc et al., 2003.

The (BM), 10-20 ml, was heparinised and mixed with a double volume of phosphate-buffered saline (PBS), centrifuged at 900g for ten minutes at room temperature, re-suspended in PBS to a density of $1x10^8$ cells/ml and layered over a 1.073 g/ml Percoll solution (Sigma-Aldrich, St Louis, MO, USA) and centrifuged at 900g for 30 minutes. The mononuclear cells were collected from the interface, washed and re-suspended in hMSC medium consisting of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Life Technologies, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Life Technologies) and plated in culture flasks (Becton Dickinson Biosciences, Bedford, MS.) at a density of 160.000 cells/cm^2 .

The serum lot was selected on the basis of optimal cell growth and differentiation. Non-adherent cells were removed after 48-72 hours and the adherent cells were cultured. Cultures were kept at 37 ^{O}C in a damp 5 % CO2 atmosphere. When >70% confluence was reached, the cells were detached by trypsin and ethylenediaminetetraacetic (EDTA) (GibcoBRL, Grand Island, NY) and replated at a density of 4000 cells/cm^2. The cells were harvested in passage five.

3.2.2 Immunocytochemistry for human mesenchymal stem cells

The hMSCs used in the studies described in paper I and III-V, were classified as mesenchymal stem cells by the flow cytometric analyses described by Le Blanc et al., 2003; i.e positive to CD73, CD90, CD105 and CD166, and negative to CD14, CD31, CD34, CD45 and CD80. The flow cytometric analyses were performed following the protocol from the manufactures respectively (Coulter, Miami, FL.; Immunotech, Marseilles, France; Becton Dickinson, San Jose, CA). Flow cytometry was carried out by collecting $1x10^4$ events on a fluorescence-activated cell sorter scan equipped with an argon laser. The data were analyzed with CELLQUEST software (Becton Dickinson, San Jose, CA).

3.3 FLUORESCENCE IN SITU HYBRIDIZATION

FISH, fluorescence In situ hybridization, has been used as a method to detect human cells in all studies, paper I-V.

A human specific probe (Red labeled total human genomic DNA, Vysis Inc., Downers Grove, IL) was used to verify the human origin of the human cells in the vocal folds.

The protocol recommended by the manufacturer was followed. The vocal fold tissue sections were de-paraffinized in xylene and rehydrated through a graded series of ethanol and water. The slides were thereafter pretreated by boiling in citrate buffer (pH 6.0) and pepsin at 37°C. Denaturation of double stranded DNA was performed by heating at 74°C for 5 minutes, and probe hybridization was allowed by overnight incubation in 37°C. temperature. The tissue sections were mounted with Vectashield containing DAPI, and analyzed using a Zeiss Axiovert 200M microscope and Openlab 5.0 as the software.

3.4 COLLAGEN TYPE I

3.4.1 Immunohistochemistry for Collagen type I staining

Paraffin embedded sections were deparaffinised in xylene, rehydrated in alcohol and blocked in PBS containing 3% BSA. Slides were incubated with a primary antibody antibody 6308, Abcam, Cambridge, UK, followed by incubation with a secondary antibody nr.A21127 Jackson ImmunoResearch labs Inc., West Grove, PA, in paper I-IV.

In paper V, antibody No 90395, Abcam, Cambridge, UK, followed by incubation with the secondary antibody nr.A11029 Alexa 488 anti-mouse, Invitrogen labs, Carlsbad, Ca. U.S. were used. The use of different antibodies was due to the fact that the former two left the market. Sections were then rehydrated in ethanol and xylene and mounted with Vectashield containing DAPI (Vector labs Inc., Burlingame, CA).

The relative contents of collagen type I in the VFs were measured from the digitized stains after a colour filtering and normalisation process with Photoshop (version 8.0) and a custom made software (Hans Larsson, Karolinska Institutet, Dept. of Logopedics and Phoniatrics).

3.4.2 Collagen type I RT-PCR

Collagen type I RT-PCR species-specific expression analysis was performed in paper II to determine whether a polyp formation in one VF was of rabbit or human origin. RNA was reversed transcribed using a Superscript III FirstStrand Synthesis System (Invitrogen Corporation) according to the manufacturer's protocol, and PCR was performed with Platinum Taq-polymerase (Invitrogen Corporation). Human specific primers (Cybergene) and conditions used were the following:

Collagen type 1: F: 5_-TTCCCCCAGCCACAAAGAGTC-3_,
R:5_-CGTCATCGCACAACACCT-3_, 50°C, 35 cycles, 261 bp.
Nanog: F:5_-CGGCTTCCTCCTCTTCCTCTATAC-3_, R:5_-
ATCGATTTCACTCATCTTCACACGTC-3_, 60°C, 30 cycles, 960 bp.

3.5 LAMINA PROPRIA THICKNESS

After fixation in 4% formaldehyde and 70% ethanol the VFs removed from the larynges were processed, dehydrated and finally embedded in paraffin wax. These blocks were cut into 5μm thick horizontal sections covering the whole thickness of each VF. Staining was made with hematoxylin and eosin (HE).

Measurements of the lamina propria (Lp) thickness were made on the digitized hematoxylin and eosin images at 10x or 20x magnification with a custom made software Hans Larsson, Karolinska Institutet. The thickest parts of the Lp were measured at three spots for each VF. If a tendency of polyp formation was seen, six measure points including the polyp were used. In Paper V six measure points were used for all samples.

3.6 VERHOEFF STAINING

Verhoeff staining was performed in paper III and IV to detect elastin. After fixation in 4% formaldehyde and 70% ethanol the VFs removed from the larynges were, dehydrated and finally embedded in paraffin wax. These blocks were cut into 5µm thick horizontal sections covering the thickness of the VF. The slides were then deparaffinised in xylene, rehydrated in alcohol and blocked in PBS containing 3% BSA.

The slides were then incubated in Verhoeff solution, containing haemotoxylin, ferric chloride and potassium iodine, for 1 hour at room temperature. Then differentiated in 2% aqueous ferric chloride for 2 minutes and subsequently treated with 5% sodium thiosulphate for 1 minute and counterstained with Van Gieson solution HT254, (Sigma-Aldrich, St Louis, MO). Finally, the slides were dehydrated, mounted and digitized at 10x or 20x magnification.

3.7 ALCIAN BLUE STAINING

Alcian blue staining was performed and studied in papers III-IV. Alcian blue staining is used to detect mucopolysaccharides and glycosaminoglycans. That is in the VFs mainly hyaluronic acid. The paraffin blocks of the VFs were cut into slides of 5µm thickness. The slides were then deparaffinised and rehydrated in series of alcohol and incubated in alcian blue stain using Alcian blue kit, #SS012, (BioGenex CA.), for 1 hour at room temperature. The slides were counterstained with nuclear fast red solution for 5 minutes. Finally, the slides were dehydrated, mounted and digitized at 10x or 20x magnification.

3.8 HEMATOXYLIN-EOSIN STAINING

The VFs were characterised into four categories depending on grade of scarring i.e. fibrosis. Grade A showed none or minimal signs of fibrosis. Grade B showed a focal or non-compact fibrosis in the Lp or superficial vocal muscle. Grade C showed a more compact fibrosis in the Lp and superficial muscle and grade D a compact fibrosis in Lp and superficial muscle as well as fibrosis in the deeper part of the vocal muscle. The method is illustrated in paper III, figure 3.

3.9 VISCOELASTIC MEASUREMENTS

In the studies described in paper I-III, the rheological measurements were performed with a parallel-plate rheometer that produced sinusoidal shear small amplitude oscillations at frequencies from 0.01 to 15Hz. An AR 2000 Rheometer (TA Instrument) was used. This rheometer had a stationary lower plate of 8 mm in diameter separated by about 0.5mm from a rotating upper plate.

Tissue samples from fresh frozen VFs were thawed in room temperature, dissected and analysed at 37°C in the parallel-plate rheometer. The samples included lamina propria and the superficial part of the thyroarytenoid muscle. The tissue was kept moisted with saline during the measurements.

All rheometric measurements were performed in the linear region with a constant strain level transferred from the sample to the upper plate where it was measured with a linear variable displacement transducer. The response and reproducibility were stable up to 2-3 Hz. For higher frequencies the results were not stable due to inertia of the measurement system probably due to the tissue samples not completely filling out the 8mm plate space.

The dynamic viscosity (η', in Pa · s) and elastic modulus (G', in Pa) were derived as a function of frequency. The absolute levels of η' and G' in paper I-III may not be accurate considering the gap between the plates not being completely filled with tissue. However, the same amount of tissue was used for all samples which allows for comparison between samples.

In the study described in paper IV the rheological measurements were carried out with a rheometer capable of measuring frequencies up to 250Hz, well into the phonatory range. A controlled-strain, linear, simple-shear rheometer based on the EnduraTEC ElectroForce 3200 mechanical testing system (Bose Corporation, ElectroForce Systems Group, Eden Prairie MN, US) was used. The rheometer was capable of empirical measurements of viscoelastic shear properties at phonatory frequencies, following previous validation (Chan and Rodriguez, 2008).

In the rheometer, a specimen was sandwiched between an upper plate and a lower plate separated by 0.5-1.0 mm. A translational displacement of a prescribed amplitude and frequency was applied to the specimen through the upper plate. The shear force resulting from the viscoelastic response of the specimen upon oscillatory shear deformation was detected by a piezoelectric force transducer attached to the lower plate. All rheometric measurements were performed in the linear viscoelastic region (at 1-2% strain) over the frequency range of 1- 250 Hz. The elastic shear modulus (G', in Pa) and the dynamic viscosity (η', in Pa · s) were derived as functions of frequency according to the theory of linear viscoelasticity (Chan and Rodriguez, 2008).

Measurements of G' (Pa) and η' (Pa · s) as functions of frequency, f, (in Hz) were plotted in log-log scale as shown in paper V figure 1a and 1b. Curve-fitting regression was then performed for each curve to examine the relationships between G' and f and between η' and f. The linear model was used for both G' and η', i.e. log (G' or η') = $B_0 + B_1 \cdot \log (f)$, where B_0 , B_1 are coefficients of parameterization. The curve-fitting estimations, based on least-squares regression analysis, were estimated using the ANOVA F test in all cases. The significant values of the F test suggested that the variation explained by the model was not due to chance. Goodness of fit was also estimated by the coefficient of determination, R^2. The R^2 statistic is a measure of the strength of association between the observed and model-predicted values for both log (G') and log (η').

3.10 VOCAL FOLD SCARRING

The rabbits laryngeal structures were visualised by means of a modified 4.0 mm paediatric laryngoscope (model 8576E, Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7 mm rigid endoscope (model 7218A). The VFs and the mobility of the cricoarytenoid joints were examined. A digital video recorded on a computer was

made of each VF before and after a scarification procedure (Richard Wolf video camera No 5512 and a Canopus ADVC100 digital video converter, Canopus Electronics Ltd., Hong Kong). The scarring procedure was performed with a 1.5 mm micro cup forceps (MicroFrance Medtronic, Düsseldorf Germany) excising the mucosa and the superficial layer of the thyroarytenoid muscle. In paper IV an established scar excision procedure was performed after nine weeks. The scar excision was performed with a 2 mm micro cup forceps (Micro France Medtronic, Düsseldorf Germany) excising all visible scarred tissue.

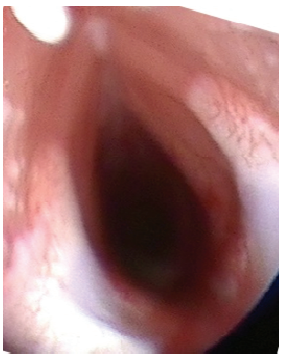

Fig. 5 Rabbit vocal fold, normal

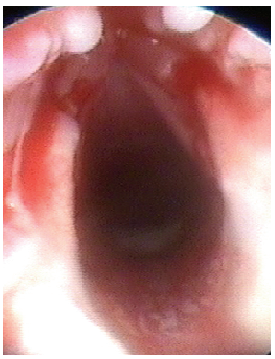

Fig. 6 Vocal fold resections

3.11 STEM CELL TRANSPLANTATION

The human stem cells were centrifuged and washed from the growth medium and suspended in saline. The solution was aspirated to fill a Medtronic Xomed laryngeal injector with a 27 gauge needle with a syringe of 1 ml. The stem cells were then transplanted into the VFs by an injection.

In the studies described in paper I-III and V, the stem cell injection was made directly after a 1.5 mm resection procedure was performed on the VF. The injection was done under video monitoring. The injection with the volume of 0.1 ml, were made into the lamina propria and/or the superficial part of the thyroarytenoid muscle of the VF. The correct injection site was stated by observed bulging of the VF corresponding to the injected volume.

In the study described in paper IV, the injection was made directly after a 2 mm scar excision procedure was done.

In paper I and in III-V human mesenchymal stem cells were used. Each VF was injected with about 80.000-100.000 human mesenchymal stem cells. In paper II human embryonic stem cells were used. About 10.000 human embryonic stem cells were injected in each rabbit VF.

3.12 IMMUNOSUPPRESSANT

In the studies analysed in paper I-IV immunosuppression was used to prevent the host versus graft reaction of the transplanted stem cells. The animals that received human stem

cells were treated with the immunosuppressant Tacrolimus (TC) 0.05mg/kg bodyweight. The TC was administrated subcutaneously every second day. The dose was based on the recommended dose/kg from the manufacturer and our previous experiments in rabbits (Hertegård et al., 2006).

The effect of the immunosuppressant Tacrolimus on mesenchymal stem cells is addressed in paper V.

3.13 ANIMALS

All the studies were carried out on the VFs of female New Zealand White rabbits with the bodyweight of 2.8 kg-4.9 kg.

They were kept in accordance with the national animal care law of Sweden and the principles of animal care of the National Institute of Health, publication 85-23.

The experiments were approved by the local ethics committee of the Karolinska Institutet, Stockholm, Sweden.

3.14 STATISTICAL ANALYSES

Kruskal-Walli´s method was used to test differences between more than two groups whereas Mann-Whitney U-test was used to analyse differences between two groups. Calculations whether or not, the dynamic viscosity and the elastic modulus, respectively, differed between groups were performed with the binomial test. In the regression analyses the F test was used. When a direction of the difference between two samples could be predicted as to their means, i.e. the mean of a sample 1 was “greater than” or “less than” that of sample 2, the test was one-tailed; otherwise it was two-tailed. For the histologic evaluation assessing grade of fibrosis, graded A-D, Fisher's Exact Probability test was used. Statistical significance was considered when $p < .05$. The statistical tests that were performed in each study are described under *Statistical analyses* in the corresponding paper I-V.

4 RESULTS AND COMMENTS

4.1 PAPER I

Human mesenchymal stem cells transplanted to injured rabbit vocal folds (VFs) engrafted and reduced scar formation with improved rheological quality of the scarred VFs - short term results of one month.

The main purposes of the study were to evaluate whether human mesenchymal stem cells (hMSCs) can engraft in the VFs of rabbits and if they do, do they improve the healing of injured VFs.

Results

Ten female New Zealand White rabbits were used in the study. Twelve VFs from 6 animals were scarred by a localized resection and the VFs were injected with hMSCs or saline. Eight VFs were left as normal controls, i.e. injected with only saline without scarring. Six VFs from 3 animals were injected with hMSCs. The left VFs were taken to histology and the right VFs to rheological measurements.

In all, 10 VFs were used for the rheological measurements; 3 hMSC treated VFs, 3VFs scarred + injected with NaCl, and 3 normal controls. The tenth VF, a normal control, was used in the calibration process of the rheological instrument and was left out.

The remaining 10 VFs were used in the histologic measurements. The lamina propria (Lp) thickness was measured on 3 hMSC treated VFs, 3 VFs scarred + injected with NaCl and 4 normal controls.

The collagen type I content measurements were carried out on 3 hMSC treated VFs (from 3 animals), 2 VFs scarred + injected with NaCl and 1 normal control.

The persistence of hMSCs after transplantation was measured after one month by the fluorescent in situ hybridization technique (FISH). The 3 hMSC-injected VFs revealed single positive human cells with a frequency of 5 to 10 cells per counted section. In the three samples an average of 11 cells per 6 thousand cells were found, which results in an engraftment of 0.18 %.

The rheological measurements also revealed that the hMSC treated VFs performed significantly lower elastic modulus (G') compared with the untreated scarred VFs. The elastic modulus was significantly lower for the normal controls than for both hMSC-treated scarred and untreated scarred VFs.

In the dynamic viscosity (η') measurements the normal controls had the lowest viscosity both when compared with the hMSC treated VFs and the untreated scarred VFs. The hMSC treated VFs showed a tendency to lower viscosity in comparison with the untreated scarred samples but no significant difference was shown.

The density of collagen type I was seen reduced in the hMSC treated VFs compared with the scarred untreated VFs. In the single normal VF analyzed, no collagen type I was found. In the two scarred untreated VFs collagen type I was richly detected.

Comments

This study showed favorable results. The results showed that human stem cells of mesenchymal origin could graft in the VFs of rabbits. It also showed that the xenograft rabbit model was a useful set up to explore the potential of hMSCs in the healing process of scarred VFs.

The study showed that the transplanted hMSCs were able not only to engraft but also survive for one month in the xenograft environment. In addition the engraftment was rather high (Liu X et al., 2011; Aicher et al., 2003).

The human cells, indicated by FISH, were found mostly in single positions. However a common finding was the presence of two nuclei in close proximity indicating mitotic activity. Larger groups of human cells were not seen. These observations indicate the possibility of a single cell division during the observation period of 4 weeks. Consequently no development of the hMSCs into derivates of muscle or cartilage was seen.

The improved elastic modulus (G') shown for the hMSC treated VFs was not due to a differentiation of the injected hMSCs. HMSCs have been shown to have immunosuppressive and anti-inflammatory properties by several investigators (Chen et al., 2008; Beyth et al., 2005; Barry et al., 2005; Le Blanc et al., 2003; Ryan et al., 2005; Krampera et al., 2003; Di Nicola et al., 2002). HMSCs have been shown to produce immunosuppressive cytokines, interleukin (IL)-10, hepatocyte growth factor (HGF), and transforming growth factor (TGF)-beta1 (Ryan et al., 2007). Tögel et al., 2005, showed in an ischemic kidney in a rat model that autologous MSCs even with a low engraftment mediated a shift in the cytokine expression profile with increased expression of anti-inflammatory cytokines and reduced renal injury.

These findings indicate the presence of a paracrine effect by the hMSCs upon the surrounding native rabbit tissue cells induced by anti-inflammatory mediators from the hMSCs. The reduced collagen type I content in the hMSC treated VFs support the assumption of an hMSC mediated reduction in the inflammation activity.

4.2 PAPER II

Human embryonic stem cells (hESCs) injected in a rabbit vocal fold at the time of injury of the VF, reduced scar formation in the VF and improved its viscoelastic properties. The hESCs also regenerated epithelium, muscle, cartilage and glands by differentiation. The injected hESCs survived one month in the rabbit VFs.

The purposes of the study were to evaluate whether hESCs can engraft in the VFs of rabbits and if they can, do they further improve the healing of an injured VF compared with hMSCs, and if engrafting do hESCs regenerate lost tissue in the scarred VF.

Results

This study consists of two repeated experiments. The experiments were conducted under the same protocol. The purpose was to verify the findings of regeneration of tissue found in the hESC treated VFs not earlier described and to compensate for lost samples in the preparation procedure in the first experiment.

In the first experiment 11 female New Zealand White rabbits were used. Out of the 22VFs 17 VFs were scarred and 5 were kept normal i.e. only injected with saline. Of the 17 scarred VFs 11 were injected with hESCs and 6 with only saline. However during the microtone cutting procedure of the specimens no less than ten samples were damaged, (5 hESC treated 2 scarred untreated and 3 normal samples).

In the second experiment 10 female New Zealand White rabbits were used. One animal died during the anesthesia before the surgical procedure was started. Of the 18 VFs 15 VFs were scarred and 11 were injected with hESCs and 4 with only saline. The remaining 3 VFs were left as normal controls i.e. injected with only saline. One of these three normal samples was then used for the calibration of the rheometer during the analysis and was left out in the calculations.

In the histologic measurements thus 17 VFs were included; 11 hMSCs treated, 4 scarred untreated controls, and 2 normal VFs, i.e. only injected with saline.

For the rheological measurements 12 VFs were included; 6 hESC treated, 4 scarred controls and 2 normal VFs, i.e. injected with only saline.

The rheological measurements revealed improved viscoelastic properties for the hESC treated VFs compared with scarred untreated VFs. The hMSC treated VFs showed significantly lower values for both elastic modulus (G') and dynamic viscosity (η') versus the untreated scarred VFs.

The histologic analyzes did not reveal any significant reduction for the hESC treated VFs compared with untreated scarred VFs in the LP thickness measurements nor in the collagen type I measurements. However, when single samples were compared the hESC treated VFs showed a less dense fibrosis, more loosely structured than in the untreated VFs.

Detection of human cells with fluorescence in situ hybridization (FISH) revealed human cells in all of the hESC treated VFs. The engraftment of the transplanted hESCs was 5.1%. A RT-PCR analysis showed the expression of human Nanog in all the VFs that had been injected with hESCs. The presence of Nanog indicates that pluripotent cells were present in the VFs one month after transplantation.

All the hESC transplanted VFs demonstrated regeneration of tissue. By fluorescence in situ hybridization (FISH) analysis the regenerated tissue was revealed to be of human origin.

All the hESC treated VFs showed new formation of human cartilage intermixed or in contact with rabbit cartilage. Human muscle tissue was detected in 45 % of the hMSC treated VFs and epithelium of human origin was found in 27 % of the hESC treated VFs.

The hESC-derived tissue areas were limited to the proximity of the injection site. This was determined by FISH analysis for human cells at different levels throughout the whole thickness of the hESC treated VFs.

One hMSC treated VF presented in the posterior part of the VF, a larger area of human origin with epithelium, muscle and also glands, all with normal histology.
No atypical tissue formations or teratomas were found in any of the hESC transplanted VFs.

RT-PCR using primers specific for human collagen was used to determine the species specificity of the collagen. The collagen was found to be of non-human origin.

Comments

This study revealed interesting results. The histologic analysis of the hESC injected VFs showed regional specific differentiation of the transplanted cells. The hESCs were differentiated into cell derivates appropriate for the environment. The hESCs were thus seen to produce areas of epithelium, muscle, cartilage and also glands as an integrated part or in close contact with the native rabbit tissue. The hESCs thus differentiated into epithelial cells, myocytes and chondrocytes.

The regenerated tissue of human origin replaced lost rabbit tissue in a structural adapting manor. The regenerated tissue was histologically fully structured and was on the hematoxylin-eosin stainings visually not separable from native rabbit tissue.

One VF presented in its posterior part a larger area of differentiated epithelium, cartilage, muscle, and glands with normal histology of human origin. The presence of glands in this area can be interpreted as an indication of human tissue expanding outside the site of injection indicating more unrestricted growth. However, native glands can be found just below the epithelial level of a VF and one or several glands might have been damaged by the biopsy at the time of scarification. If so, the presences of glands represents adequate regeneration and rather points to the regenerative capacity of the hESCs.

The human derived tissue was localized in the area of injection and was not seen to migrate or become unstructured nor form teratomas. However two hESC treated VFs showed polyp formations as a sign of excessive fibrosis. This was reflected in the

collagen measurements where these VFs presented a collagen density well comparable with untreated scarred VFs. But the other hMSC treated VFs showed generally minor, loose and less spread fibrosis compared with the untreated scarred VFs. Though considering this observation the collagen measurements of the hESC treated VFs did not show any significant reduction in collagen type I content compared with the untreated scarred VFs. This stands in contrast with the finding from the study of human mesenchymal stem cells (hMSCs) in Paper I, where the transplanted hMSCs reduced the collagen type I content in the hMSC treated VFs.

This however is in consistency with other findings. Embryonic stem cells have been found to be allogeneic and induce a similar immune response as human fibroblast cells when transplanted and to be acutely rejected in a xenogeneic setting of mice (Grinnemo et al., 2006). This is in contrast with the hMSCs that are known to avoid allorecognition and to generate a local immunosuppressive microenvironment (Ryan et al., 2005, 2007).

The collagen type I in the hESC transplanted VFs, was traced by RT-PCR using primers specific for human collagen. The collagen in the hESC transplanted VFs, was shown to be of non-human origin. It has been shown that an increased collagen synthesis in an in vitro culture of murine embryonic stem cells (ESCs) stimulated the ESCs to differentiate into mesodermal tissue (Sato et al., 2006). The increased level of collagen or rather non-significant decrease of collagen in the hESC treated VFs, might have induced the rapid differentiation and growth of the transplanted hESCs.

The hESC treated VFs showed significantly reduced both elastic modulus (G') and dynamic viscosity (η') compared with the untreated scarred VFs. The non-reduced content of collagen type I does not seem to have influenced the viscoelastic improvement induced by the transplanted hESCs. This supports the observation that the fibrosis seen in the hESC treated VFs was in general less dense more diffuse than in the untreated scarred VFs. This indicates that the synthesized collagen might be more structured and less bundle like in the hESC treated VFs than in the untreated scarred VFs, thus being less compromising to the rheological properties of the VF.

The hESCs survived one month in the rabbit VFs and moreover some of them preserved their pluripotent state.

4.3 PAPER III

Human mesenchymal stem cells injected in a rabbit vocal fold at the time of injury of the VF, reduced scar formation in the VF and improved its viscoelastic properties. The improvements were sustainable for three months. The injected hMSCs did not survive three months in the VFs of rabbits.

The purposes of the study were to evaluate if the results of improved histologic and viscoelastic properties found in hMSC treated scarred rabbit VFs after one month, were repeatable and sustainable over a longer time, and if the injected hMSCs survived three months in the VFs of rabbits.

Results

Eleven female New Zealand White rabbits were used in the study. 18 VFs from 9 animals were scarred by a localized resection and the VFs were injected with hMSCs or saline. Four VFs were left as normal controls, i.e. injected with only saline without scarring. Ten scarred VFs from 5 animals were injected with hMSCs.

Ten VFs were used for the rheological measurements; 3 hMSC treated VFs, 3VFs scarred + injected with NaCl, and 4 normal controls.

Twelve VFs were taken to histology. Five normal controls, injected with only saline without scarring, were added from a databank to the histological analyzes. Thus 17 VFs were included in the histologic analyzes; 7 hMSC treated VFs, 5 VFs scarred + injected with NaCl, and 5 normal controls.

The persistence of hMSCs after transplantation was measured after three month by FISH. About 10^4 cells per VF injected with hMSCs were counted. No human cells were detected.

The rheological measurements revealed that the hMSC treated VFs performed significantly lower elastic modulus (G') compared with the untreated scarred VFs, and moreover the hMSC treated VFs showed no significant difference compared with normal VFs.

Treatment with hMSCs was also found to significantly decrease the dynamic viscosity (η') compared with untreated controls and was not significantly higher than for normal VFs.

In the histologic analyses the hMSCs showed significantly reduced values for both collagen type I content and the Lp- thickness compared with untreated scarred VFs. Moreover, no significant differences were shown in collagen type I content or in Lp-thickness between hMSC treated VFs and normal VFs.

From the Hematoxylin-eosin stainings the VFs were visually characterized into four categories depending on grade of fibrosis. There was a tendency though not significant, that the hMSC treated VFs were placed in the A and B groups representing less scarring

and the untreated VFs in the C and D groups, indicating more general fibrosis. (The classification principles are described in *MATERIAL AND METHODS*)

Verhoeff staining for elastin and alcian blue staining for hyaluronic acid were also performed but revealed no significant differences.

Comments

The results of the study confirmed the findings from the one month analyses of paper I. The VFs that received transplanted hMSCs showed improved viscoelasticity compared with the untreated VFs. The elastic modulus (G') that was found significantly reduced for the hMSC-treated VFs compared with untreated after one month was found significantly reduced also after 3 months.

The dynamic viscosity (η') that showed a tendency to be reduced by treatment with hMSCs after one month was found significantly reduced compared with untreated scarred VFs. The finding that there were no significant differences in elastic modulus (G') or dynamic viscosity (η') between normal VFs and hMSCs treated VFs is an extremely interesting finding.

The histological results referring to the collagen type I content and the Lp thickness, mirrored the viscoelastic measurements. The collagen type I density, which was found significantly reduced after one month for the hMSC treated VFs compared with the untreated scarred VFs, stayed so also after three months. In addition the Lp-thickness was found significantly reduced in the hMSC treated VFs compared with the untreated. Like in the viscoelastic measurements there were no significant differences in neither of the collagen type I nor in the Lp-thickness measurements, when normal VFs and hMSC treated VFs were compared.

The findings that there were no significant differences in elastic modulus (G') or dynamic viscosity (η') nor in collagen type I content or the Lp thickness between normal VFs and hMSCs treated VFs, are indeed encouraging. However, it is important to bear in mind that the hMSC treated VFs were not equal to the normal VFs when the epithelium, lamina propria and the vocal muscle were visually analyzed regarding a general appearance of fibrosis. In the hMSC treated VFs there were a general tendency to thicker epithelium and slight unevenly expressed fibrosis in the Lp and in the superficial part of the thyroarytenoid muscle. This is not found in normal VFs.

At the endpoint of the study at three months no FISH stained human cells were detected. The transplanted hMSCs do not seem to survive three months in the xenograft environment of rabbits.

Like in the one month study (paper I) no human tissue was seen. This indicates that the improvements in viscosity and histology of the hMSC treated VFs are not caused by a differentiation of the implanted hMSCs but by a direct stimulation on the cells in the surrounding of the transplanted hMSCs. This paracrine effect can influence the resident cells in the injured VF or influence the chemotactically attracted inflammatory cells to modify the healing process in the scarred VF. The hMSCs have been shown to stimulate

the production of a numerous of growth factors, cytokines and chemokines (Nauta and Fibe, 2007; Le Blanc and Ringdén, 2007; Uccelli et al., 2008; Pati et al., 2011; Liu X et al., 2011).

4.4 PAPER IV

Human mesenchymal stem cells injected in a rabbit vocal fold after a scar excision, reduced Lp-thickness and restored the viscoelasticity of the VF.

The main purpose of this study was to evaluate if the improved healing observed at one and three months post injection with hMSCs in injured rabbit VFs, could be transferred to a clinic like model by excising an established scar and thereafter transplant hMSCs into the scar excised VF.

Results

Twelve female New Zealand White rabbits were enrolled in the study. Twenty (20) VFs from 10 animals were scarred by a localized resection and were then left to heal with scar formation. After 9 weeks all visible scar tissue was resected from the scarred VFs. An injection transplanting hMSCs to the wound bed were made directly after the scar excision procedure. After another 10 weeks the VFs were analyzed for their rheological and histological properties.

Fourteen VFs were injected with hMSCs. Six VFs were injected with saline. Four VFs were left as normal controls, i.e. injected with only saline without scarring.

Fourteen VFs were used for the rheological measurements; 7 hMSC treated VFs, 3VFs scarred and injected with NaCl, and 4 normal controls.

Ten VFs were taken to histology. Five normal controls, injected with only saline without scarring, were added from the databank to the histological analyses. Thus 15 VFs were included in the histological analyses. All 15 VFs were used in the collagen type I measurements; 7 hMSC treated VFs, 3VFs scarred and injected with NaCl, and 5 normal controls. In the Lp- thickness measurements two VFs in hMSC treated group was lost during the preparation procedure, resulting in 5 hMSC treated VFs, 3 untreated scarred VFs and 5 normal controls for the measurements.

In the rheological measurements the scarred untreated VFs presented for the two viscoelastic parameters, dynamic viscosity (η') and elastic modulus (G') significantly increased values compared to normal VFs. The VFs transplanted with hMSCs showed significantly decreased values for both dynamic viscosity and elastic modulus compared with the untreated scarred VFs. The hMSC treated VFs showed no significant difference in dynamic viscosity (η') nor in elastic modulus (G') when compared to normal VFs.

In the histologic analyses the hMSCs treated VFs showed significantly reduced thickness of the Lp in comparison with untreated scarred VFs. No significant difference was found in the Lp-thickness between normal VFs and hMSC treated VFs.

The collagen type I measurements revealed no significant differences in density between hMSC treated VFs and untreated scarred VFs. Neither was there any significant difference between normal and hMSC treated VFs. A significant difference between normal and scarred untreated VFs was noted.

In order to visually grade whether a general fibrosis in the VF was present, the VFs were designed to four groups depending on the general fibrosis seen in the Hematoxylin-eosin stainings. There was no significant differences in the group classification between the hMSC treated VFs and the untreated scarred VFs. However, a clear tendency was observed with most of the hMSC treated VFs aligned to the two groups with least fibrosis and the untreated scarred VFs to the two lower groups representing more general fibrosis.

FISH analysis was performed to determine the persistence of hMSCs. No human cells were detected.

Comments

This study showed considerably favorable results. The results show that the positive findings found in the studies of one and three months duration (Paper I and III) are substantially repeatable and moreover transferable to a clinic like approach.

The VFs that received transplantation with hMSCs showed improved viscoelasticity compared with the untreated scarred VFs. Both the elastic modulus (G') and the dynamic viscosity (η') were found to be significantly reduced in the hMSC treated VFs. The finding that there were no significant differences in elastic modulus (G') nor in dynamic viscosity (η') between normal VFs and hMSCs treated VFs revealed at three months post injury, (paper III) were found to be repeatable in this study. Considering that two operations were performed in this study and the possibility of not all microscopic scarred tissue being excised at the scar excising procedure, the viscoelastic results are very interesting findings.

The viscoelastic results in this study shall also be seen in the light of being performed in the extended frequency range up to 250 Hz, i.e. at phonatory frequencies.

The histologic measurements of the Lp-thickness reflected the viscoelastic results. The hMSC treated VFs showed no significant difference compared with normal VFs but showed a significant reduction when compared to untreated scarred VFs.

However the collagen type I content measurements, that at one and three months showed a significant reduction for the hMSC treated VFs compared with the untreated scarred VFs, did not display any significances in this study except between normal and untreated scarred VFs.

As in the study of three months duration (paper III), a general diffuse though parsimonious fibrosis in the hMSC treated VFs was observed. This was recognised on the Hematoxylin-eosin stainings. The hMSC treated VFs tended to show a slightly thicker epithelium and sparse unevenly expressed fibres in the Lp somewhat reaching in between the muscle bundles in the most superficial part of the thyroarytenoid muscle. This general though sparse mellow fibrosis seems to have been reflected in the collagen type I measurements but did not significantly influence the viscoelastic parameters.

At the endpoint, 10 weeks post transplantation of the hMSCs, no human cells were detected by FISH. The transplanted hMSCs do not seem to survive 10 weeks in the rabbit xenograft environment.

4.5 PAPER V

The immunosuppressant Tacrolimus used in the rabbit xenograft model to reduce the host versus graft reaction, did not contribute to the improved healing of the scarred rabbit vocal folds but suppressed the anti-scaring effect of the transplanted human mesenchymal stem cells.

The purpose of the study was to evaluate if the improved healing described for scarred rabbit VFs after hMSC or hESC transplantation (paper I-IV), can be attributed to the immunosuppressant Tacrolimus and not to the stem cells. Human MSCs were used in the study. The duration of the study was one month.

Results

Ten female New Zealand White rabbits were used in the study. The 20 VFs from the 10 animals were scarred by a localized resection and the VFs were injected with hMSCs or saline. Eighteen (18) VFs were injected with hMSCs. Two (2) VFs were injected with only saline.

Four of the animals whose VFs received injection with hMSCs (i.e. 8VFs), and the animal whose VFs were injected with only saline (i.e. 2VFs) were treated subcutaneously with the immunosuppressant, Tacrolimus (TC) every second day. The dose was identical with our previous experiments.

Four VFs from a previous pilot study, one identically treated with the hMSC group and one with the hMSC+TC group and two with the TC group, were included in the analyses of grade of fibrosis and Lp-thickness. Additional data for 5 normal VFs were collected from the data bank from earlier experiments. The analyses of grade of fibrosis and Lp-thickness were thus carried out on, 11 hMSC treated, 9 hMSC+TC treated, 4 TC treated and 5 normal VFs.

For the relative collagen type I measurements the samples from the pilot study could unfortunately not be included as different collagen probes were used in the studies. Another probe had to be used as the original one had gone out of production. This also meant that no normal VFs were at hand for comparison. One VF in the hMSC group and 2 in the hMSC+ TC group failed to mark in properly and were for this reason left out.The collagen type I analyzes were thus performed on 9 hMSC treated, 6 hMSC +TC treated, and 2 TC treated VFs.

In the Lp measurements the hMSC treated VFs revealed a significant reduction in the Lp thickness compared with both hMSC+TC and TC treated VFs. The hMSC+TC treated VFs presented a thinner LP than the TC treated VFs. Noticeable was that the hMSC treated VFs exhibited a significantly thicker LP than normal VFs.

The relative collagen type I analyses showed significantly less density for the hMSC treated VFs compared with both hMSC+TC treated and only TC treated VFs. No significance was found between hMSC+TC treated and TC treated VFs.

When the Hematoxylin-eosin stainings of the VFs were visually characterized into four categories depending on grade of general fibrosis there was significantly less fibrosis in the hMSC treated VFs compared with the TC treated VFs. No significant differences were explored between hMSC treated versus hMSC+TC treated nor between hMSC+TC versus TC treated VFs. Furthermore there were no significant difference between hMSC treated and normal VFs. The difference between normal and TC treated VFs was significant.

FISH-analysis for the survival of the transplanted hMSCs was both with and without the immunosuppressant TC, 0.09% engraftment.

Comments

This study showed favorable results for the hMSCs. The results showed that the immunosuppressant Tacrolimus did not contribute to the improved healing seen in the hMSC transplanted scarred rabbit VFs but significantly reduced the anti-scaring effect of the transplanted hMSCs.

In the Lp thickness measurements both the TC treated and the hMSC+TC treated VFs presented thicker Lp than the VFs that received only hMSCs. These results show that when TC is present the effect of the hMSCs in reducing the Lp thickness is restrained.

In the classification of the grade of general fibrosis the hMSC treated VFs produced significantly less fibrosis than the TC treated VFs. However there were no significant difference between the hMSC+TC and the TC treated VFs. These observations clearly show that the grade of fibrosis is reduced by the transplanted hMSCs. The lack of difference between the hMSC+TC and TC treated VFs indicates in the light of a significant difference present between hMSC treated and TC treated VFs a clear repression upon the anti- scaring effect of the hMSCs.

The results referring to the collagen type I content showed significantly less density for the hMSC treated VFs compared with both hMSC+TC treated and the VFs treated with only TC. That hMSC treated VFs expressed reduced collagen compared with the hMSC+TC treated VFs, strongly confirm the presence of a repression on the hMSC mediated effect to reduce collagen in the scarred VFs. That hMSC+TC treated VFs compared with TC treated VFs did not show any significant reduction further supports the assumption that the hMSCs were hampered by the immunosuppression.

In this study no comparisons were performed between untreated scarred VFs and scarred TC treated VFs. The question whether the immunosuppressant per se expresses some anti-inflammatory or scar diminishing effect has not been assessed in this study.

However the results strongly support the assumption that the immunosuppressant Tacrolimus, used in the studies in the xenograft model (Paper I-IV), did not contribute to the reduction of the fibrosis seen in the hMSC treated scarred rabbit VFs but suppressed the effect of the hMSCs.

This study investigated the impact of the immunosuppressant Tacrolimus on hMSCs transplanted to scarred VFs in a xenograft model of rabbits. The negative impact of the TC on the effects mediated by the hMSCs seems reasonable to convey also to the effect of the hESCs (Paper II).

With an engraftment of .09 % for both hMSC and hMSC+TC treated VFs, no impact upon the survival of the transplanted hMSCs was detected in this study.

5 CONCLUSIONS

The following conclusions can be drawn based on the findings in the thesis

- Both human embryonic (hESCs) and mesenchymal (hMSCs) stem cells can engraft in the vocal folds (VFs) of rabbits.

- HESCs can survive and maintain their pluripotent state for one month in rabbit VFs.

- HMSCs can survive one month but do not seem to survive ten weeks in the VFs of rabbits.

- A transplantation of hESCs into injured rabbit VFs improves the viscoelastic properties of the VFs at one month.

- HESCs have the capacity to regenerate lost tissue in scarred rabbit VFs, by differentiation into epithelium, muscle, and cartilage compatible with the native rabbit tissue.

- HMSCs transplanted into injured rabbit VFs reduce scar formation and render improved viscoelastic properties to the VFs at one month. The improvements are persistent at three months.

- The potency of hMSCs to reduce scar formation and improve the viscoelastic properties of a rabbit VF after an acute injury, is transferable to a potentially clinic model of excising a chronic scar followed by an hMSC transplantation.

- HMSCs do not seem to differentiate in the VFs of rabbits.

- The immunosuppressant Tacrolimus suppresses the ant-scaring effect of transplanted hMSCs.

Altogether the results presented in the thesis indicate that both human embryonic and mesenchymal stem cells have the potential to improve healing and to restore the rheological properties of scarred vocal folds.

6 FUTURE PERSPECTIVES

The embryonic stem cells have the greatest potential for tissue regeneration. The hESCs have shown capacity to rapidly evolve into the cell types of epithelium, muscle cartilage and glands in scarred rabbit VFs. The finding of persistent undifferentiated hESCs in the rabbit VFs after one month demands further investigation. Although no atypical tissues or teratoma formations were seen in this study (paper II), it is needed to reveal the destiny of these pluripotent cells in a longer term.

Being pluripotent the hESCs harbor the risk of developing not only teratomas but also teratocarcinomas (Solter, 2006). The risk of malignancies has so far challenged the clinical use of embryonic stem cells.

Furthermore, the ethical concerns of how embryonic stem cells are originally derived have raised doubt, which has hampered both the preclinical and clinical research on embryonic stem cells (Passier et al., 2008).

Until the risk of maligification and the ethical concerns have been fully assessed the use of hESCs will likely be restrained to preclinical research.

The transplanted mesenchymal stem cells however, did not show any signs of differentiation in the studies (paper I and III-V). This is an important finding and an essential knowledge when evaluating the safety of hMSCs for clinical use.

The positive paracrine effect of the transplanted hMSCs in scarred rabbit VFs has been clearly revealed in the studies (paper and III-IV). The mechanisms behind this paracrine effect from the hMSCs on the environment in the VFs though, are not clear and challenge further investigation.

However, the results of this thesis in particular those of the study described in paper V, encourage to a clinical trial using autologous mesenchymal stem cells.

7 SVENSK SAMMANFATTNING

Kirurgi och strålbehandling av stämband läker ofta med ärrbildning i det vibrerande lagret av stämbandet dvs. lamina propria (Lp). Ärrskador i ett stämbands Lp orsakar uttalade röstproblem. Ärrskador i stämband är mycket svårbehandlade. Någon effektiv behandlingsmetod för att läka ärr i stämband finns ej. De material som idag används för injektion eller implantation, såsom hyaluronan och fett, fyller ut det defekta stämbandet, men läker det inte. För att en läkning skall kunna ske utan ärr krävs en minskad inflammatorisk reaktion samt en regeneration av de förlorade vävnadskomponenterna i stämbandet. Stamceller har visats ha potential att minska inflammation och regenerera vävnad. En stamcells regenerativa förmåga kan förväntas bero på dess differentieringsgrad.

En xenograft modell med kanin har utvecklats för att utvärdera humana mesenkymala och embryonala stamcellers läknings potential.

Projektet har omfattat 5 delstudier.

Studie I: Studie avseende humana mesenkymala stamcellers (hMSC) potentiella förmåga att reducera ärrläkningen i sårskadade stämband. Tio New Zealand White kaniners stämband sårgjordes med en 1,5mm vid biopsitång. HMSC injicerades i direkt anslutning till skadan. Efter en månad analyserades stämbandens histologi, lamina propria tjocklek, relativ halt av kollagen typ I, viskoelasticitet i vävnaden samt engraftment (grad av överlevnad av transplanterade celler). De hMSC behandlade stämbanden visade signifikant förbättrade viskoelastiska egenskaper och minskad kollagenhalt jämfört med de obehandlade stämbanden. HMSC kärnor iakttogs intill varandra i de med hMSC injicerade stämbanden indikerande mitos aktivitet. Ingen human vävnad kunde påvisas.

Studie II: Studie avseende humana embryonala stamcellers (hESC) potentiella förmåga att reducera ärrläkningen i sårskadade stämband. Identiskt studieupplägg med studie 1. HESC linje HS181 (Hovatta et al 2003) användes. Fyrtio stämband från kaniner ingick i studien. I alla stämband som injicerades med hESC påvisades engraftment. I de hESC injicerade stämbanden påvisades öar av humant epitel, muskel, och brosk i omedelbar anslutning till värddjurets eget epitel, muskel resp. broskvävnad. Viskoelasticitets mätningar visade signifikant förbättrade biomekaniska egenskaper för de hESC injicerade stämbanden jämfört med obehandlade stämband.

Studie III: Tre månaders studie av ärrskadade kaninstämband injicerade med humana mesenkymala stamceller (hMSC) med frågeställningarna; är de förbättrade viskoelastiska egenskaperna efter injektion med hMSC som påvisades efter en månad, bestående? Överlever engraftade hMSC 3 mån? Elva New Zealand White kaniner ingick studien. Viskoelasticitetsmätningar visade bestående förbättring i förhållande till obehandlade stämband och ingen signifikant skillnad jämfört med normala stämband. Reduktionen i

lamina proprias tjocklek och halten av kollagen typ I var signifikant sänkt för de hMSC behandlade stämbanden jämfört med de obehandlade. Inga hMSC överlevde 3 månader.

Studie IV: I studierna I-III har stamceller injicerats i omedelbar anslutning till en iatrogent åstadkommen sårskada i ett friskt stämband. Denna studie avsåg att studera effekten av stamcells behandling av en etablerad ärrskada. Tolv New Zealand White kaniner ingick studien. En lokaliserad sårskada åstadkoms i stämbanden, men dessa lämnades sedan till självläkning. Efter 9 veckor exciderades den bildade ärrvävnaden och hMSC injicerades i stämbanden. Efter ytterligare 10 veckor analyserades stämbanden avseende histologi och viskoelasticitet. De hMSC behandlade stämbanden visade viskoelastiska egenskaper i paritet med normala och signifikant bättre än obehandlade.

Studie V: I studierna I-IV har immunosuppression, Tacrolimus (TC) använts för att förhindra avstötning av de humana stamcellerna. Det skulle därför kunna tänkas att TC i sig har bidragit till den förbättrade läkningen i de stamcells behandlade stämbanden. Tio New Zeeland White kaniner ingick i studien. HMSC injicerades i 18 sårgjorda stämband, 2 injicerades med enbart koksalt. Hälften av djuren gavs TC subkutant. Därefter utvärderades histologi avseende grad av fibros, lamina propria tjocklek och kollagen typ I halt i vävnaden. TC behandling medförde signifikant sämre läkning i samtliga parametrar jämfört med hMSC behandling. TC befanns hämma hMSC positiva effekt på läkningen av stämbanden.

Slutsatser: Både humana embryonala och mesenkymala stamceller har förmåga till engraftment i en xenomodell med kanin.

Både humana embryonala och mesenkymala stamceller läker sårskadade kanin stämband signifikant bättre än obehandlade, avseende viskoelasticitet analyserat efter 1 respektive 3 månader.

Humana embryonala stamceller regenererade i de ärrskadade stämbanden efter en månad humant epitel, muskel och brosk i anslutning till värddjurets egen motsvarande vävnad.

I en klink liknande modell för excision av ärrvävnad och behandling med transplantation av humana mesenkymala stamceller visade stamcellerna att de läker de sårgjorda stämbanden i xenomodellen, avseende viskoelasticitet och Lp-thickness, till i paritet med normala stämband och signifikant bättre än obehandlade stämband.

Immunosuppression, Tacrolimus (TC) givet i samtliga studier motverkade stam cellernas effekt.

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Viscoelastic and Histologic Properties in Scarred Rabbit Vocal Folds After Mesenchymal Stem Cell Injection

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Objective/Hypothesis: **The aim of this study was to analyze the short-term viscoelastic and histologic properties of scarred rabbit vocal folds after injection of human mesenchymal stem cells (MSC) as well as the degree of MSC survival. Because MSCs are anti-inflammatory and regenerate mesenchymal tissues, can MSC injection reduce vocal fold scarring after injury?** ***Study Design:*** **Twelve vocal folds from 10 New Zealand rabbits were scarred by a localized resection and injected with human MSC or saline. Eight vocal folds were left as controls.** ***Material and Methods:*** **After 4 weeks, 10 larynges were stained for histology and evaluation of the lamina propria thickness. Collagen type I content was analyzed from six rabbits. MSC survival was analyzed by fluorescent in situ hybridization staining from three rabbits. Viscoelasticity for 10 vocal folds was analyzed in a parallel-plate rheometer.** ***Results:*** **The rheometry on fresh-frozen samples showed decreased dynamic viscosity and lower elastic modulus ($P < .01$) in the scarred samples injected with MSC as compared with the untreated scarred group. Normal controls had lower dynamic viscosity and elastic modulus as compared with the scarred untreated and treated vocal folds ($P < .01$). Histologic analysis showed a higher content of collagen type 1 in the scarred samples as compared with the normal vocal folds and with the scarred folds treated with MSC. MSCs remained in all samples analyzed.** ***Conclusions:*** **The treated scarred vocal folds showed persistent MSC. Injection of scarred rabbit vocal folds with MSC rendered improved viscoelastic parameters and less signs of scarring expressed as collagen content in comparison to the untreated scarred vocal folds.** ***Key Words:*** **Vocal fold scarring, vocal fold, viscoelasticity, mesenchymal stem cells.**

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INTRODUCTION

Vocal fold scarring may have different etiology such as trauma, surgical defects of the vocal folds, postradiotherapy, or inflammation.[1] This results in tissue defects and/or disturbed viscoelastic properties of the vocal fold lamina propria (increased stiffness). Voice is often breathy or aphonic and the phonation threshold pressure, which corresponds to "easiness of phonation,"[2] is elevated. The treatment is usually difficult and may include voice therapy by a speech and language therapist and injection augmentation. Many substances have been tried. Bovine or autologous human collagen has been used for superficial injections into the vocal fold ligament.[3,4] Autologous fat implantation into the lamina propria has also been tried in selected cases.[5] Drawbacks with collagen and fat are the need for allergy testing (for bovine collagen) and the unpredictable degree of resorption over time (for both).[6] Hyaluronan injections have been shown to improve viscoelastic properties in animal experiments in rabbits and improved glottal vibrations and voice of selected patients with scarring or sulcus vocalis.[7,8] Other tissue-engineering approaches to treat vocal scarring is by injection of growth factors or cells with a potential to regenerate the lamina propria. Hirano et al. reported improved viscoelastic properties as well as less scar tissue in scarred larynges after hepatocyte growth factor injection.[9] Chhetri et al. implanted autologous fibroblasts after a scarring procedure in canine larynges and showed im-

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proved vibratory characteristics but increased fibroblasts, collagen, and reticulin and a decreased density of elastin.[10]

Postnatal bone marrow contains mesenchymal stem cells (MSCs) capable of differentiating into bone, adipose, cartilage, and myelosupportive stroma in vitro and in vivo.[11–13] MSCs have immunomodulatory and possibly also antiinflammatory properties and suppress alloreactive T-cell responses.[14,15] Human MSCs reduce the proliferation of both human and murine lymphocytes.[16] Intravenous administration of MSCs improves the outcome of renal, neural, and lung injury in experimental animal models mainly through paracrine effects and a shift from proinflammatory to antiinflammatory cytokines at the site of injury.[17–19] After allogeneic stem cell transplantation, infusion of MSC reversed steroid-resistant graft-versus-host disease.[20] Kanemaru et al. injected MSC after a scarring procedure in canine and rat vocal folds.[21,22] Injected folds showed normalized macroscopic appearance after 2 months in contrast to the defect healing (e.g., atrophy, granuloma formation) found in untreated control folds.[21] However, no functional testing, for example, rheology, was performed. A later study showed that the stem cells survived and differentiated in the implanted rat vocal folds.[22]

One aim of this study was to investigate the short-term viscoelastic properties of scarred rabbit vocal folds after injections of MSCs as compared with scarred vocal folds injected with saline. Vocal fold mucosa from noninjected rabbit larynges served as controls. Another aim was to study the survival of the injected human MSC. A third aim was to analyze the degree of scarring in the lamina propria of scarred MSC-treated, untreated, and control folds.

MATERIALS AND METHODS

Ten New Zealand white rabbits (2.9–3.2 kg) were used in the experiment. The American principles of laboratory animal care and the Swedish national law on animal care ethics were followed. The experiment was approved by the local ethics committee of Karolinska Institute (S-201, November 24, 2003).

Vocal Fold Scarring

After premedication with glycopyrrolate (0.1 mg/kg subcutaneously) and fluanizonum (10 mg/mL fentanyl 0.3/mg/mL, 0.3 mL/kg diazepam, 0.3 mL/kg intramuscularly), all animals were anesthetized with 1 to 2 mg/kg diazepam intravenously. The laryngeal structures and the mobility at the cricoarytenoid joints were found normal at examination by means of a modified 4.0-mm pediatric laryngoscope (model 8576E; Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7-mm rigid endoscope (model 7218A). A video recording on the computer was made of the vocal folds before and after the scarification procedure (Panasonic video camera and a Capture to Go video card, version 1.18; Margi System Inc., Fremont, CA). The scarring procedure was performed with a 2-mm microcup forceps and microscissors (MicroFrance Medtronic, Düsseldorf, Germany). A localized excision of the mucosa and superficial thyroarytenoid muscle was made under video monitoring (Fig. 1). The procedure yielded 12 excised (scarred) vocal folds and eight normal vocal folds without scarring; at least one vocal fold was scarred in each animal. All animals survived the procedure.

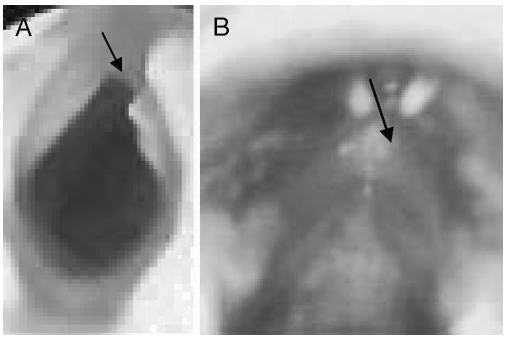

Fig. 1. Right vocal fold has a defect after scarring procedure (arrow left image); right picture after 1 month. The arrows mark the right vocal fold, which was injected with human mesenchymal stem cell.

Mesenchymal Stem Cell Preparation and Characterization

MSCs were isolated and expanded from bone marrow (BM) taken from the iliac crest of healthy volunteers as previously described.[15] The procedure was approved by the ethics committee at Karolinska University Hospital Huddinge. Heparinized BM was mixed with a double volume of phosphate-buffered saline (PBS), centrifuged at 900 *g*, resuspended, and layered over a Percoll gradient (1.073 g/mL; Sigma-Aldrich, St. Louis, MO). The mononuclear cells were collected from the interface, washed, and resuspended in human MSC medium consisting of Dulbecco modified Eagle's medium–low glucose (DMEM-LG; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Life Technologies). The serum lot was selected on the basis of optimal MSC growth and differentiation. The cells were plated in culture flasks (Becton Dickinson Biosciences, Bedford, MS) at a density of 160,000 cells/cm^2. Nonadherent cells were removed after 48 to 72 hours and the adherent cells were cultured. When >70% confluence was reached, the cells were detached by trypsin and ethylenediaminetetraacetic (EDTA; GibcoBRL, Grand Island, NY) and replated at a density of 4000 cells/cm^2. The cells were harvested in passage 5 and classified as MSCs based on their ability to differentiate into bone, fat, and cartilage and by flow cytometric analysis (positivity for CD73, CD90, CD105, and CD166; negativity for CD14, CD31, CD34, CD45, and CD80).

Vocal Fold Injections

The MSC were centrifuged and washed from the growth medium in NaCl. Injections were made under video monitoring directly after the scarification procedure into the lamina propria and/or to the superficial part of the thyroarytenoid muscle of the vocal fold using a Medtronic Xomed laryngeal injector with a 27-gauge needle. Systematic injections in either of the structures mentioned were not possible because of the narrow space and the equipment available at the time of the experiment. Six of the 12 scarred vocal folds were injected with MSC in 0.1 mL saline each. Each vocal fold was injected with approximately 80,000 MSCs. Six vocal folds were injected with 0.1 mL saline. The eight nonscarred vocal folds were not injected. No animal experienced breathing problems or bleeding after the injections. The animals that received MSCs were treated with a low dose of immunosuppressant (0.07 mg/kg tacrolimus body weight subcutaneously) every second day during 1 month to prevent rejection. The dose was estimated from previous experience of experiments in rats.[23]

Moreover, the general status and well-being of the rabbits as well as appetite and weight was monitored to avoid overdosing the immunosuppression. In some cases, when an animal tended to lose weight, the tacrolimus dose was postponed 1 day. All animals kept their weight and no signs of side effects of the immunosuppression were noted during the month of observation.

Dissection

Four weeks after the injections, the animals were killed by an intravenous overdose of sodium pentobarbital. The larynges were dissected out and each larynx was divided in the posterior midline. Ten of the hemilarynges were immediately fresh-frozen at −20°C until viscoelastic analysis (four noninjected, three scarred vocal folds injected with saline, three scarred vocal folds injected with MSC).

Ten of the hemilarynges were placed in 4% formaldehyde for later preparation and histologic analysis (four noninjected, three scarred folds injected with saline, three scarred folds injected with MSC).

Histologic Measurements

The vocal folds removed from the hemilarynges were further processed in 4% buffered formalin solution rinsed in 0.1 mol/L phosphate buffer. The vocal folds were then decalcified in 10% EDTA at 37°C in a microwave oven set at 450 W for 3 hours, rinsed in 0.1 mol/L phosphate buffer, and dehydrated in graded series of ethanol, and finally embedded in paraffin wax and cut into 5-μm thick sections. Stains were made with hematoxylin & eosin for histologic analysis. Image analysis on the stains at 20× or 40× magnification was made after digitization of the microscopic images (using a Nikon Digital Camera, DXM 1200 attached to a Nikon Ecclipse, E600 microscope) (Fig. 2). The thickness of the lamina propria was measured with the software Image Pro Plus (version 3.0; Media Cybernetics).

Collagen staining, immunohistochemistry. Paraffin-embedded sections were deparaffinized in xylene and dehydrated in ethanol. Blocking was performed in PBS for 1 hour. Slides were incubated with a primary monoclonal mouse antibody against collagen type 1 (antibody 6308; Abcam, Cambridge, U.K.) followed by incubation with a secondary fluorescent Cy-3-labeled general antibody against mouse (Jackson ImmunoResearch Labs Inc., West Grove, PA). The sections were washed in PBS containing Tween-20 and BSA-c and mounted with Vectashield containing DAPI (Vector Labs Inc., Burlingame, CA).

The relative content of collagen type 1 in the vocal folds was measured from the digitized stains after a color filtering and normalization process with Photoshop (version 8.0) and custom-made software (written by Hans Larsson at Karolinska Institute, Stockholm, Department of Logopedics and Phoniatrics). In total, collagen type 1 stainings from six different animals were analyzed.

Fluorescent in situ hybridization analysis for persistence of transplanted human mesenchymal stem cells. Paraffin-embedded sections were deparaffinized in xylene and dehydrated in ethanol. Slides were pretreated in citrate buffer and incubated in 0.01 mol/L HCl containing 1% pepsin at 37°C. The slides were washed in NP40 and PBS and dehydrated in ethanol. The slides were air-dried and human-specific fluorescent-labeled probe (SpectrumRed human genomic DNA; Vysis Inc., Downers Grove, IL) was applied to the sections. Probe and target DNA were denatured simultaneously at 74°C and allowed to hybridize overnight in a humidified chamber at 38°C. Slides were washed in NP40 at 72°C. The sections were dried and mounted with Vectashield containing DAPI (Vector Labs Inc.).

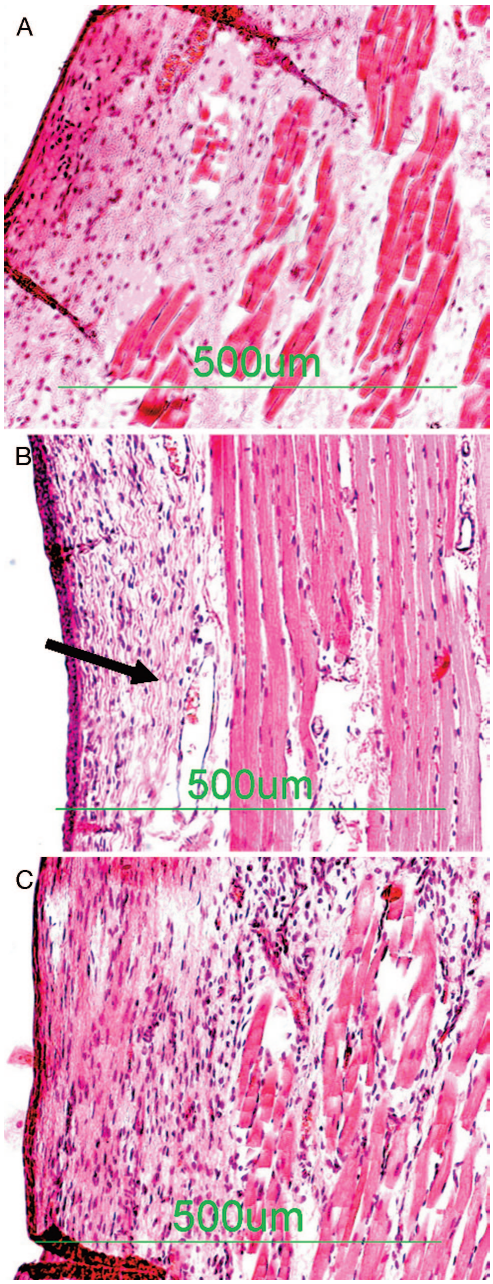

Fig. 2. Hematoxylin & eosin staining of the membranous parts of vocal folds. (A) Normal, (B) scarred vocal fold injected with saline (arrow marks bundles in lamina propria), and (C) scarred vocal fold injected with mesenchymal stem cells (×20).

Viscoelastic Measurements

Parallel-plate rheometry. The linear viscoelastic shear properties of vocal fold tissue have been studied by several researchers.[24,25] The parallel-plate rheometer in this experiment produces sinusoidal shear small-amplitude oscillations at increasing frequency (from 0.01–15 Hz). We used an AR 2000 Rheometer (TA Instrument) with a stationary lower plate (8-mm diameter) separated by approximately 0.5 mm from a rotating upper plate. Tissue samples from the nine freshly frozen vocal folds were thawed in room temperature, dissected, and analyzed at 37°C in the parallel-plate rheometer (three untreated, three scarred fold injected with saline, and three scarred folds injected with stem cells). One untreated sample was used to calibrate the equipment and the results are not included in the analyses. The samples included vocal fold lamina propria and the superficial part of the thyroarytenoid muscle. The tissue was kept moist with saline during the measurements. All rheometric measurements were performed in the linear region with constant strain level transferred from the sample to the upper plate where it was measured with a linear variable displacement transducer. In this experiment, the response and reproducibility was stable up to approximately 3 Hz. For higher frequencies, the results were not stable probably as a result of inertia of the measurement system probably because the tissue samples were not geometrically perfectly flat and did not completely fill out the 8-mm plate space. The dynamic viscosity (η', Pas) and elastic modulus (G', Pa) were derived as a function of frequency. Dynamic viscosity is a measure of a material's resistance to shear flow. The elastic (storage) modulus (G') represents a measure of a material's stiffness in shear. As mentioned in this experiment, the gap between the plates was not completely filled with tissue. Thus, the absolute level of η' and G' may not be accurate. However, the same dissection procedure and amount of tissue was used for all samples, which allow for comparison between the different treatment groups.

Statistics

Nonparametric comparisons between the groups were made (Statview program, SAS Institute Inc., version 5.0) for measurements of the dynamic viscosity and the elastic modules; Wilcoxon paired comparison of mean values were used for all frequencies. As a result of the exploratory nature of the study, significance levels with $P < .05$ are reported.

RESULTS

Histologic Analyses

A normal untreated control rabbit vocal fold has an amorphous lamina propria with no longitudinal collagen-like bundles visible (Fig. 2A). An untreated scarred vocal fold has visible longitudinal collagen-like fiber bundles in the lamina propria (Fig. 2B). In contrast, less bundles were found in folds injected with MSC (Fig. 2C). In general, the visible differences between the hematoxylin & eosin-stained samples were small with a thickness of the lamina propria of 200 to 250 μm. The density of collagen type 1 was higher in the vocal folds (thyroarytenoid muscle and lamina propria) of the untreated scarred samples than in the normal controls and the scarred folds injected with MSC (Fig. 3). Of the six different animals we analyzed, three animals injected with MSC had collagen content of 1.5% (in Fig. 3), 2.0% and 2.7% of the total slide area, respectively. Two animals with untreated scar had collagen content of 2.9% and 7.3% (shown in Fig. 3). The last animal was a normal control in which no collagen could be quantified (in Fig. 3).

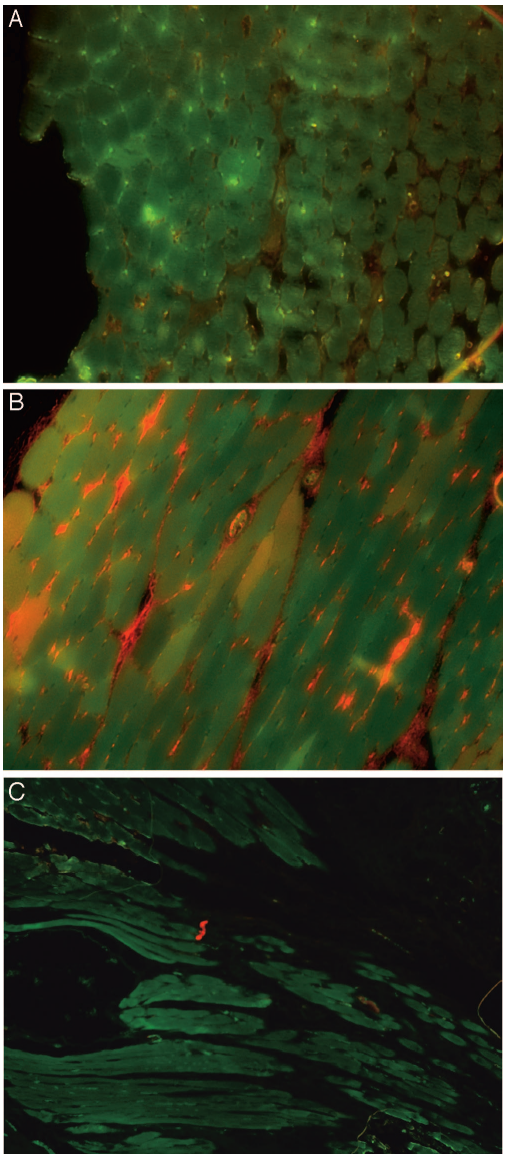

Fig. 3. Detection of collagen type 1 by immunofluorescence (×20) of the midmembranous vocal fold part of (A) normal, (B) scarred untreated, and (C) scarred fold injected with mesenchymal stem cell.

Persistence of Mesenchymal Stem Cell After Transplantation

Sections of the dissected hemilarynges were hybridized to a human-specific fluorescent-labeled probe and

analyzed for the presence of human cells. No signal was detected in a negative control using noninjected rabbit vocal folds (data not shown). A slide from a human teratoma was used as positive control (not shown).

MSC-injected vocal folds in three separate rabbits were investigated. Fluorescent in situ hybridization (FISH) analysis of three hemilarynges sections from each vocal fold revealed single positive human cells with a frequency of five to 10 cells per section. A common finding was the presence of two nuclei in close proximity indicating mitotic activity (Fig. 4). Larger groups of human cells were not detected, consistent with only one cell division occurring during the observation period of 4 weeks. Six thousand cells were counted in randomly chosen sections of vocal folds treated with MSC. In total, 11 human cells were found, resulting in 0.18% engraftment.

Viscoelastic Analyses

Dynamic viscosity (η', Pas). Normal controls had the lowest viscosity both when compared with the untreated scar larynges and the scarred samples treated with MSC (normal:scar $P < .01$, normal:MSC $P < .01$) (Fig. 5A). The scarred larynges treated with MSC showed a tendency to lower viscosity than the untreated scar samples (MSC:scar $P = .07$).

Elastic modulus (G', Pa). The elastic modulus was significantly lower for the normal controls than for both the untreated and MSC-treated scar samples (normal:scar $P < .01$, normal:MSC $P < .01$) (Fig 5B). The vocal folds treated with MSC had significantly lower elastic modulus than the untreated scar folds (MSC:scar $P < .01$).

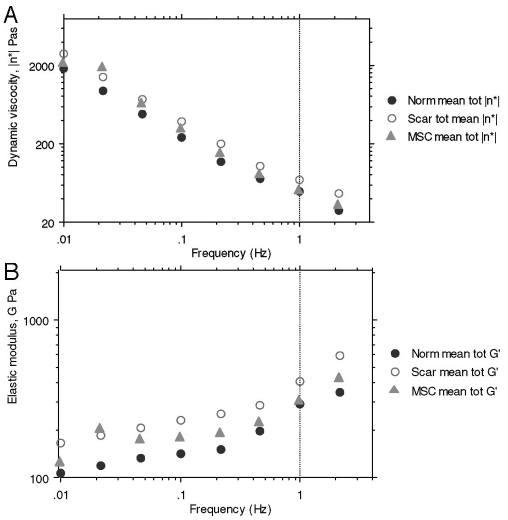

Fig. 5. (A) Graph of the dynamic viscosity (η', Pas) as a function of oscillatory frequency of the parallel-plate rheometer for the controls (normal:mean, n = 3), scarred fold injected with saline (scar:mean, n = 3), and scarred folds injected with mesenchymal stem cell (MSC:mean, n = 3). (B) Graph of the elastic modulus, G', as a function of oscillatory frequency of the parallel-plate rheometer for the controls (normal:mean, n = 3), scarred fold injected with saline (scar:mean, n = 3), and scarred folds injected with MSC (MSC: mean, n = 3).

DISCUSSION

At present, there is no effective method for prevention or treatment of vocal fold scarring, although some researchers have presented promising results, for example, with hepatocyte growth factor injections[9] or hyaluronan injections.[7,26]

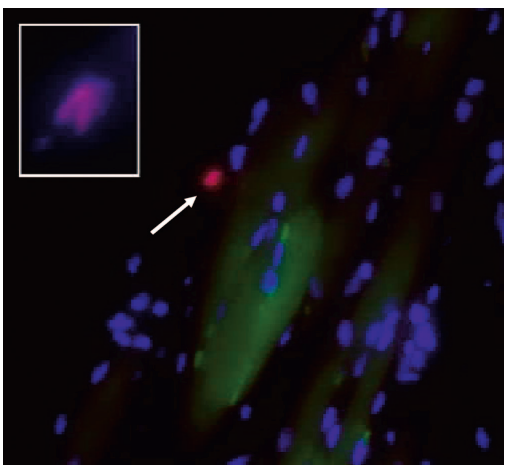

Fig. 4. The midmembranous vocal fold of a rabbit injected with human mesenchymal stem cell was hybridized with a probe for the human genome (red) with DAP1 staining of the nucleus (×40). The arrow indicates dividing cell (enlarged top left).

This study shows favorable results for scarred vocal folds treated with human MSC both regarding the viscoelastic properties (functional result) and the histologic picture. Our results corroborate the histologic findings of Kanemaru et al.[21,22] In the present study, the untreated larynges had increased collagen type and viscoelastic data indicating increased stiffness as a result of scarring. The viscoelasticity in the scarred samples treated with MSC was higher than in the normal controls but significantly lower than in the untreated scar samples, indicating that MSC improved healing. The exact mechanism of this is not clear, but vocal fold scarring and stiffness has been attributed to an increased collagen content of the lamina propria.[27] The collagen type 1 content of the vocal folds was lower in the MSC-treated than in the untreated scarred folds. In several experiments, the effect of bone marrow-derived MSC for treatment of bleomycin-induced lung fibrosis in mice was studied.[17,28] Rojas et al. found that MSCs were important in the repair of bleomycin-injured lung and that MSCs protect against injury.[28] The MSC localized to the injured lung and assumed lung cell phenotypes, but protection from injury and fibrosis also involved suppression of inflammation and an increased production of reparative growth factors. Similarly, MSC administration reduced bleomycin-induced inflammation

and collagen deposition in lung tissue.[17] A recent study of ischemic acute renal failure showed that, although engraftment was low, MSC mediated a shift in the cytokine expression profile with increased expression of antiinflammatory cytokines and reduced renal injury.[18]

The FISH analysis of the present study indicates survival and division of MSC in the vocal folds. In the studies of Kanemaru et al.,[21,22] histologic analysis also showed evidence of MSC survival in the vocal folds as well as evidence of differentiation into at least two laryngeal cell types in injected nude rats: epithelial and muscle.[22] We did not study the cell differentiation pattern of the MSC in this experiment. It is possible that MSCs participate in the healing process as they produce matrix molecules, including laminin and collagen, and express integrins that constitute receptors for extracellular matrix components.[29] Young et al. previously showed that autologous MSC improved healing of injured rabbit Achilles tendons.[30] Also, after implantation into cartilage defects, MSC engrafted and secreted collagen type 2 that was gradually replaced by type 1 collagen.[31]

In this study, rabbits treated with human MSC were given low-dose immunosuppression (tacrolimus). Neither undifferentiated nor differentiated human MSC stimulate alloreactivity in vitro and fully HLA-mismatched allogeneic MSC persisted long term after transplantation into an immunocompetent fetus in the third trimester of gestation.[32,33] Instead, human MSCs are immunosuppressive in vitro and reduce lymphocyte proliferation and the formation of cytotoxic T cells and dendritic cells.[29] MSCs also prolong the time to rejection of histoincompatible skin grafts and may be used clinically to reverse severe acute graft-versus-host disease after allogeneic stem cell transplantation.[20,34] However, these immunosuppressive mechanisms may not be sufficient to prevent rejection in a discordant xenogeneic setting. Although MSC suppress lymphocyte formation, immune responses after xenotransplantation include both acquired and innate immunity in which natural antibody, complement, natural killer cells, and macrophages all play independent roles.[35] Human MSCs were rejected in a xenogeneic model after implantation into Sprague Dawley rats.[36] However, even in immunocompetent RNU rats, engraftment was improved in animals treated with tacrolimus.[23] However, if infused in utero to fetal sheep or in the brain of albino rats, human MSCs persist long term.[13,37] As a result in part of their relatively closed compartment localization, it is possible that vocal folds act as an immunoprivileged site explaining persistence of human cells in this xenogenic setting. Species-specific differences may also exist as immunologic tolerance to mouse MSC in rats was reported by Saito et al. and could explain engraftment of human MSC in rabbits but not in rats.[38] Only MSC-treated rabbits received tacrolimus. Thus, there is a possibility that tacrolimus improved healing rather than the MSC. However, we consider it unlikely that immunosuppression is beneficial to the healing of vocal folds and that it is rather attributed to the MSC. Our findings are corroborated by Rojas et al.[28] They found that myelosuppression increased the susceptibility to bleomycin injury but that MSC infusion was protective.

CONCLUSION

We have shown survival of human MSC with potential in vivo cell division after injection into scarred rabbit vocal cords. As measured by parallel-plate rheometry, MSC treatment improved biomechanical properties in scarred folds and reduced the collagen type 1 content. A prolonged follow-up time, between 3 and 6 months, may be required to fully determine the stable collagen type 1 content and functional results. Such studies are currently underway and include investigation of other factors and molecules that may be important to vocal fold function.

Acknowledgments

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II

The Laryngoscope
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Injection of Embryonic Stem Cells Into Scarred Rabbit Vocal Folds Enhances Healing and Improves Viscoelasticity: Short-Term Results

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Objectives: Scarring caused by trauma, postcancer treatment, or inflammation in the vocal folds is associated with stiffness of the lamina propria and results in severe voice problems. Currently there is no effective treatment. Human embryonic stem cells (hESC) have been recognized as providing a potential resource for cell transplantations, but in the undifferentiated state, they are generally not considered for therapeutic use due to risk of inadvertent development. This study assesses the functional potential of hESC to prevent or diminish scarring and improve viscoelasticity following grafting into scarred rabbit vocal folds.

Study Design: hESC were injected into 22 scarred vocal folds of New Zealand rabbits. After 1 month, the vocal folds were dissected and analyzed for persistence of hESC by fluorescence in situ hybridization using a human specific probe, and for differentiation by evaluation in hematoxylin-eosin-stained tissues. Parallel-plate rheometry was used to evaluate the functional effects, i.e., viscoelastic properties, after treatment with hESC.

Results: The results revealed significantly improved viscoelasticity in the hESC-treated vs. non-treated vocal folds. An average of 5.1% engraftment of human cells was found 1 month after hESC injection. In the hESC-injected folds, development compatible with cartilage, muscle and epithelia in close proximity or intermixed with the appropriate native rabbit tissue was detected in combination with less scarring and improved viscoelasticity.

Conclusions: The histology and location of the surviving hESC-derived cells strongly indicate that the functional improvement was caused by the injected cells, which were regenerating scarred tissue. The findings point toward a strong impact from the host microenvironment, resulting in a regional specific in vivo hESC differentiation and regeneration of three types of tissue in scarred vocal folds of adult rabbits.

Key Words: Human embryonic stem cells, xenograft, rabbit, vocal fold, differentiation.

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INTRODUCTION

Vocal fold scarring is the most common cause of severe voice disturbances.[1,2] The causes may be trauma, postcancer treatment, inflammation, or congenital. This leads to decreased viscoelasticity of the vocal fold lamina propria, which reduces the vibration potential of the vocal folds. In vocal fold scarring, the voice is often severely dysphonic or aphonic and phonation is strained. Currently there are no effective treatment methods. Recent research focusing on injection augmentation of molecules in the extracellular matrix (e.g., collagen, hyaluronic acid, etc.) has led to several important findings. Hyaluronan is thought to be important for the vocal fold function and injection may improve viscoelasticity in scarring.[3–6] Kanemaru et al. injected autologous mesenchymal stem cells (MSC) after a scarring procedure in canine and rat vocal folds.[7] Injected folds showed normalized macroscopic appearance after 2 months in contrast to the defective healing (e.g., atrophy, granuloma formation) found in untreated control folds. A later study showed that the stem cells survived and differentiated in the implanted rat vocal folds.[8] In a recent experiment, we injected human MSC into scarred rabbit vocal folds and found a low degree of survival of the transplanted MSC. However, healing was

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improved as compared to non-treated scarred vocal folds with less collagen type 1 content in the lamina propria and improved viscoelasticity of the vocal fold tissue.[9]

Since the first description of successful in vitro culture of hESC, such cells have been recognized as providing a potential resource for cell transplantations.[10] Here, we examined the survival and development of undifferentiated hESC injected into scarred rabbit vocal folds with the intention to assess their functional potential to prevent or diminish scarring. Others have reported on similar approaches to verify the functional capacity of hESC and their cell derivatives for functional regeneration of specific tissues in vivo. Muotri et al.[11] performed xenografts of hESC into mouse embryonic brain and found support for hESC being responsive to environmental signals and to acquire both morphologic and functional differentiation with regional specificity. Goldstein et al.[12] found that hESC transplanted into or in place of epithelial-stage somites of chick embryos of 1.5 to 2 days of development integrated well, and that this environment may modulate hESC differentiation. Both these studies showed a regional impact on hESC differentiation after xenografting to an embryonic environment, mouse[11] and chick,[12] respectively. Our study addresses and extends this notion to adult tissue in the rabbit and demonstrates a region-specific hESC differentiation into muscle, cartilage, and epithelium leading to a measurable functional restoration of scarred vocal folds.

MATERIALS AND METHODS

Ethical Permission

This study was performed with the permission of the Local Ethics Committee at Karolinska Institutet (114/00) and by the Regional Committee of South Stockholm for Animal Experimentation (S-172-03 and S-224-04).

Cells

The hESC line HS181(46;XX),[13] derived by the hESC-network at Karolinska Institute was maintained as previously described on mitotically inactivated (35 Gy irradiation) human foreskin fibroblasts.[14] HS181 cells corresponding to passage 32 from two separate thawings were used in this study.

Xenograft Model

Two similar experiments using female New Zealand white (NZW) rabbits were performed (the body weight of rabbits in experiment one was 4.6 to 4.9 kg, and in experiment two was 2.9 to 3.2 kg).

Experiment one included 11 rabbits. Of the total 22 vocal folds, 5 were not operated ("untreated control"), 6 were operated with a scarring procedure only ("scarred untreated control"), and 11 vocal folds were scarred and injected with hESC ("scarred hESC injected"). Experiment two included 10 rabbits, but 1 died during the anesthesia before the scarring procedure. Thus, of the 18 vocal folds in experiment two, 3 were used as untreated controls, 4 were scarred untreated controls, and 11 were scarred hESC injected.

Vocal Fold Scarring

After premedication with glycopyrrolate (0.15 mg/kg subcutaneously) and Hypnorm (fentanyl 0.3/mg/mL mixed with fluanizonum 10 mg/mL, 0.3 mL/kg IM), all animals were anesthetized with diazepam 2 mg/kg IV. The laryngeal structures and the mobility at the cricoarytenoid joints were found normal by examination using a modified 4.0 mm pediatric laryngoscope (model 8576E, Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7 mm rigid endoscope (model 7218A), i.e., no scarring or vocal fold immobility. A digital video recording on computer was made of the vocal folds before and after the scarring procedure (Richard Wolf video camera No 5512, and Canopus ADVC100 digital video converter, Reading, U.K.). The scarring procedure was performed with a microcup forceps and microscissors, resulting in a wound dimension approximately corresponding to 2 mm, the size of the forceps (Micro-France Medtronic, Düsseldorf, Germany). The localized excision of the mucosa and superficial thyroarytenoid muscle was made under video monitoring (Fig. 1A).

hESC Injections Into Scarred Vocal Folds

Directly after the scarring procedure, injections were made under video monitoring into the lamina propria and/or to the superficial part of the thyroarytenoid muscle of the vocal fold using a Medtronic Xomed laryngeal injector. Three to four undifferentiated hESC colonies (approximately 10^4 cells) were dissected directly from the culture plates by a 27-gauge needle, carefully avoiding the surrounding feeder cells, and then injected using the same syringe.

In total (both experiments), 22 of the 32 scarred vocal folds were injected with hESC (10^4 cells in 0.1 mL saline). Ten vocal folds were injected only with 0.1 mL saline. Eight non-scarred vocal folds were used as controls. No animal suffered from breathing problems or bleeding after the injections. All hESC-injected animals were treated with a low dose of immunosuppressant (tacrolismus 0.05 mg/kg bw subcutaneously) every second day during 1 month.[9] All animals maintained body weight, and no signs of side effects from the treatments were noted during the observation period.

Dissection of Vocal Folds

In both experiments, the animals were sacrificed 4 weeks after the injections using an IV overdose of sodium pentobarbital. The larynges were dissected out and each larynx was divided in the posterior midline. Twenty-eight of the hemilarynges (all 22 from experiment one and 6 from experiment two) were stored in 4% formaldehyde for later preparation and histologic analysis (6 untreated controls, 6 scarred controls, and 16 scarred hESC injected). Twelve of the 18 hemilarynges from experiment two were immediately fresh-frozen at –20°C prior to viscoelastic analysis (2 untreated controls, 4 scarred controls, and 6 scarred hESC injected).

Histologic Measurements

After fixation in 4% formaldehyde, the vocal folds removed from the hemilarynges were further processed, dehydrated, and finally embedded in paraffin wax and cut into 5-μm thick sections.[9] Sectioning was made throughout the whole thickness of the vocal fold. The sections corresponding to the vocal fold level where the scarring was performed were then stained using hematoxylin-eosin for histologic analysis. Digitization of the microscopic images was followed by image analysis at 20× or 40× magnification using a Nikon digital camera, DXM 1200, attached to a Nikon Eclipse, E600 microscope (Tokyo, Japan). Measurements of the mean lamina propria thickness were made with the software Image Pro Plus (version 3.0, Media Cybernetics, Bethesda, MD).

Viscoelastic Measurements

Viscoelastic measurements were performed using parallel-plate rheometry. The linear viscoelastic shear properties of vocal

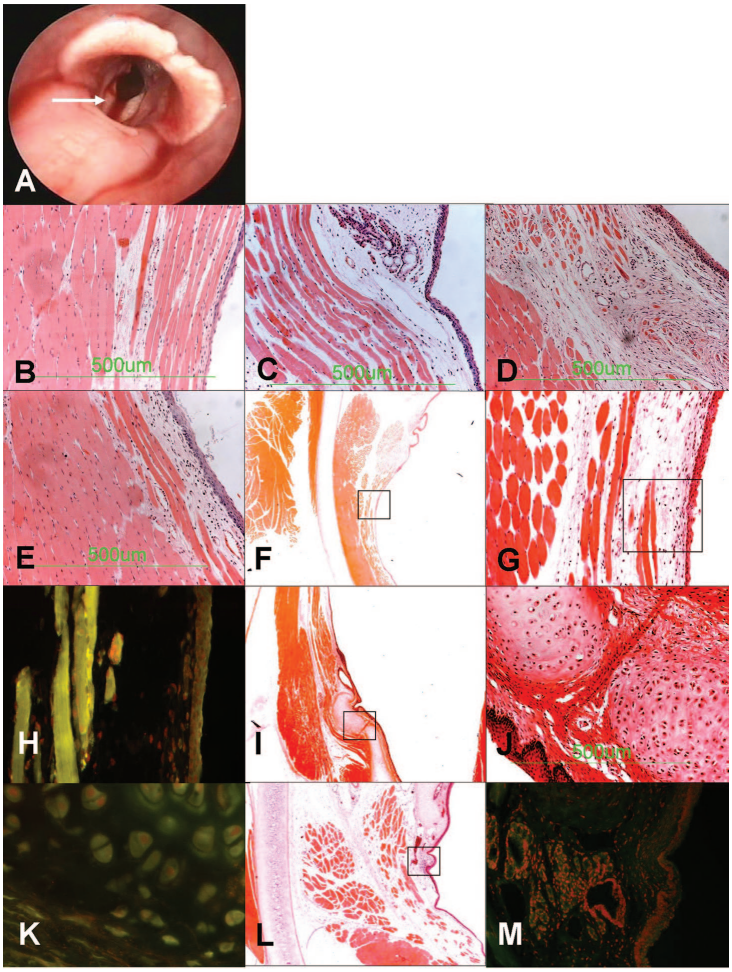

Fig. 1. Image from a rabbit larynx with a surgical scar defect (arrow) in the right vocal fold (A). hematoxylin-eosin (H&E)-staining of sections from a normal vocal fold (B); scarred vocal fold with polyp formation (C); scarred vocal fold with longitudinal collagen-like fiber bundles (D); and scarred vocal fold injected with human embryonic stem cells (hESC) (E); all at 20× magnification. (F–M) Sections of scarred hESC-injected vocal fold with (F) area compatible with differentiation into muscle and epithelium, H&E-stained: 2× magnification; (G) 20× magnification of the boxed area in F; (H) 40× magnification of area in neighboring section (equivalent to the boxed area in G stained with a nuclear fluorescent red probe (fluorescence in situ hybridization [FISH]) for human cells; (I) area compatible with differentiation into cartilage, H&E-stained: 2× magnification. (J) 20× magnification of the boxed area in I; (K) 40× magnification of area in neighboring section stained with a nuclear fluorescent red probe (FISH) for human cells; (L) area compatible with differentiation into human cartilage, muscle, epithelium, and human glands, H&E-stained 10× magnification; (M) 40× magnification of area in neighboring section (equivalent to boxed area in L) stained with a nuclear fluorescent red probe (FISH) for human cells. The nuclear localization of red signals if H, K, and M were verified by counterstaining with DAPI (not included in the picture for clarity). Green autofluorescence can also be seen.

fold tissue have been previously studied by several researchers.[15,16] An AR 2000 Rheometer (TA Instrument, New Castle, DE) with a stationary lower plate (8-mm diameter) separated by about 0.5 mm from a rotating upper plate was used. This parallel-plate rheometer produces sinusoidal shear, small amplitude oscillations at increasing frequency (0.01–15 Hz). Tissue samples from freshly frozen vocal folds were thawed, dissected, and analyzed at 37°C in the parallel-plate rheometer. The control samples were used to calibrate the equipment (data not shown). All rheometric measurements were performed in the linear region with a constant strain level transferred from the sample to the upper plate where it was measured with a linear variable displacement transducer. The response and reproducibility of several frequency sweeps were stable up to about 3 Hz. The dynamic viscosity ($|n^*|$, Pa.s) and storage (elastic) modulus (G', Pa) were derived as a function of frequency. Dynamic viscosity is a measure of a material's resistance to shear flow; the elastic (storage) modulus (G') represents a measure of a material's stiffness in shear. In the results presented, the absolute levels of η' and G' may not be fully accurate since the gap between the plates could not be completely filled with tissue. However, the same dissection procedure and amount of tissue was used for all samples, and this allows comparison between the different treatment groups.

Paraffin Removal and RNA Extraction for RT-PCR

Paraffin-embedded tissue was deparaffinized for RNA extraction and expression analysis with RT-PCR. Sections of paraffin-embedded tissue were treated with octane/methanol, precipitated by centrifugation, and homogenized using Trizol (Invitrogen Corporation, Foster City, CA) in accordance with the manufacturer's protocol.

RT-PCR

Species-specific expression analysis was performed for collagen type 1, and also for Nanog, a transcription factor

considered responsible for pluripotency and a marker for hESC. Expression of human Nanog would thus indicate persistence of pluripotent hESC.

RNA was reversed transcribed using a Superscript III First Strand Synthesis System (Invitrogen Corporation) according to the manufacturer's protocol, and PCR was performed with Platinum Taq-polymerase (Invitrogen Corporation). Human specific primers (Cybergene) and conditions used were the following: Collagen type 1: F: 5′-TTCCCCCAGCCACAAAGAGTC-3′, R:5′-CGTCATCGCACAACACCT-3′, 50°C, 35 cycles, 261 bp. Nanog: F:5′-CGGCTTCCTCCTCTTCCTCTATAC-3′, R:5′-ATCGATTTCACTCATCTTCACACGTC-3′, 60°C, 30 cycles, 960 bp.

Immunohistochemistry

FISH Analysis for Persistence of Transplanted hESC

Detection of human cells with fluorescence in situ hybridization (FISH) analysis was performed as previously described.[9] Briefly, slides were deparaffinized in xylene and rehydrated in a series of alcohol, followed by pretreatment with pepsin. Genomic DNA and probe were denatured simultaneously by heating at 74°C for 8 minutes. Hybridization was performed overnight at 38°C with a human-specific fluorescent probe (SpectrumRed human genomic DNA, Vysis Inc., Downers Grove, IL), which tested negative in non-hESC injected rabbit vocal folds, ensuring that the probe is not cross-reacting with cells of rabbit origin. Finally, the slides were mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA). Sections from 3 to 5 levels of the vocal fold thickness for each sample were used for FISH analysis to determine the engraftment.

Collagen Staining

This was performed on sections immediately surrounding hESC positive sections (as detected by FISH).

Staining was performed as previously described.[9] Briefly, slides were deparaffinized in xylene, rehydrated in a series of alcohol, and blocked in phosphate buffered solution (PBS) containing 3% bovine serum albumin (BSA). Slides were incubated with a primary antibody diluted 1:200 (antibody 6308, Abcam, Cambridge, UK), followed by incubation with a Cy3-conjugated secondary antibody diluted 1:400 (antibody A21127, Jackson ImmunoResearch Labs Inc., West Grove, PA). Sections were rehydrated in ethanol and xylene and mounted with Vectashield containing DAPI (Vector Laboratories, Inc.).

The relative content of collagen type 1 in the vocal folds was measured from the digitized stains after a color filtering and normalization process using Adobe Photoshop (version 8.0, Adobe, San Jose, CA) and custom-made software (Hans Larsson, Karolinska Institutet, Department of Logopaedics and Phoniatrics, Stockholm, Sweden). One image (corresponding to the level of FISH analysis) from each of the 13 animals was evaluated (2 untreated controls, 4 scarred controls, and 7 scarred hESC injected).

Statistics

Non-parametric comparisons (Statview, version 5.0, SAS Institute Inc., Cary, NC) between the groups were made. For measurements of the dynamic viscosity and the elastic modules, Wilcoxon's paired comparison of mean values was used for all frequencies. Due to the exploratory nature of the study, significance levels with $P < .05$ are reported.

RESULTS

Histologic Analysis

Measurements of the thickness of the lamina propria (lp) were performed on 2 untreated controls (Fig. 1B), 4 scarred untreated (Fig. 1C, D), and 11 scarred hESC-injected vocal folds (Fig. 1E). The scarred untreated vocal folds had the thickest lp with a of mean 210 μm (SD 178); scarred hESC-injected folds had a mean of 130 μm (SD 79); and the untreated control samples had a mean of 120 μm (SD 97) (nonsignificant differences).

The samples from the scarred untreated group all showed polyps or thick collagen bundles in the lp (Fig. 1C, D). The scarred hESC-injected group had polyps in only 2 of 11 samples, and thus showed less deviation regarding lamina propria structure and thickness as compared to the untreated controls group (0 of 2 with polyps; Fig. 1B).

Collagen Type 1 Content

Seven scarred hESC-injected vocal folds had relative collagen contents of between 4.2 and 25.2%, respectively, of the total slide area, while the four scarred untreated samples showed collagen contents between 5.0 and 8.5%. The untreated control vocal fold samples had collagen type 1 content of 1.7% and 4.6%, respectively. With one exception (one animal in the scarred hESC-injected group), no significant difference in collagen type 1 levels was detected when the different groups were compared. Notably, this sample (with the highest relative collagen content; 26%) was one of the very rare cases (2 of 11) with polyp formation in this group (see Histologic Analysis).

The collagen type 1 in the hESC-injected group was not of human origin as detected by RT-PCR using primers specific for human collagen (data not shown).

Persistence, Differentiation, and Engraftment of hESC After Transplantation

The persistence of human cells 4 weeks after hESC injection was evaluated with the use of FISH and fluorescence-labeled human reference DNA. The presence of hESC-derived cells in the host tissue 4 weeks after injection was demonstrated by detection of FISH-positive cells in 11 of 11 scarred hESC-injected vocal folds. For an evaluation of the degree of engraftment, 6000 FISH-positive cells were counted in chosen sections from all scarred hESC-injected vocal folds. The results showed a mean engraftment of 5.1% (an average of 306 FISH-positive cells / 6,000 cells analyzed, N = 11).

Histologic analysis of hESC-injected vocal folds revealed regional specific differentiation of human cells into types of tissues normally seen in vocal folds, i.e., cartilage, muscle, and epithelium (shown in Figs. 1F–K). All the hESC-injected areas (in 11 of 11 scarred hESC-injected vocal folds) showed a similar formation of human cartilage intermixed with rabbit cartilage. In addition, in 3 of these 11 scarred hESC-injected vocal folds, areas compatible with the formation of human cartilage were detected in immediate proximity to the native rabbit cartilage, but clearly separated from the existing cartilage. Human muscle was detected in 5 of 11, and epithelium of human origin was found in 3 of 11 scarred hESC-injected vocal folds. The hESC-derived areas were generally limited to the immediate injection location. However, in 1 of the 11 scarred hESC-injected vocal folds, the posterior part of the lamina propria consisted of a larger area of human origin, not only differentiated into cartilage, muscle,

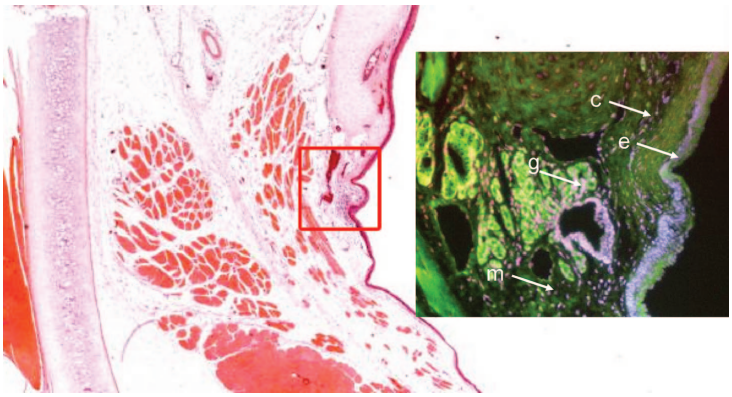

Fig. 2. Derivation of human cartilage (c), epithelia (e), glands (g), and muscles (m) from the injected human embryonic stem cells (hESC) shown in hematoxylin-eosin-staining (5× magnification). The human origin of the cells is detected by fluorescence in situ hybridization (FISH) using a red probe (counter-staining with DAPI results in pink staining of the human cells).

and epithelium, but also into glands with normal histology (Fig. 1M and Fig. 2). This vocal fold also contained a small area with subepithelial lymphocytes of rabbit origin, indicating a local cellular immune reaction despite the immunosuppressive treatment.

Expression of Nanog

Results from the RT-PCR revealed expression of human Nanog in all tested vocal folds injected with hESC, indicating the presence of pluripotent cells after 1 month.

Viscoelastic Analyses

This analysis is illustrated in Figure 3. The group of scarred hESC-injected larynges showed significantly lower dynamic viscosity (η', Pas) than did the scarred untreated samples ($P = .03$). Scarred hESC-injected vocal folds had a significantly lower elastic modulus (stiffness) than the scarred untreated folds did ($P = .03$).

DISCUSSION

Three types of tissues were regenerated at the site of hESC injection. Following what appears as a regional specific in vivo differentiation of the injected hESC, areas compatible with cartilage, human muscle, and epithelium development were observed as integrated parts of, or in close contact to, the appropriate native rabbit tissues of the vocal folds. Except for this immediate regeneration of tissue originating from the injected human cells, it cannot be excluded that the hESC or its derivatives may also have a paracrine effect on the surrounding tissue. From the FISH analysis for human cells at different levels throughout vocal fold thickness, it was clear that human cells were detected in close proximity to the injection site. Larger areas of human tissue, expanding outside the site of injection and indicating what could be perceived as more unrestricted growth, was detected in only 1 of the total 22 hESC-injected vocal folds. In this case, most of the posterior part of the lamina propria was found to consist besides human cartilage, muscle, and epithelium, also of tissue compatible with human glands, however, without any apparent signs of anaplasia or resemblance of teratoma formation. Somewhat surprising, and given reservations of performing and evaluating a complete histopathologic analysis, all analyzed samples of the human tissues formed were judged as histopathologically normal. Areas with cells of malignant phenotype could not be detected within the observation period of 1 month. Long-term studies following the hESC transplantation, where cells have

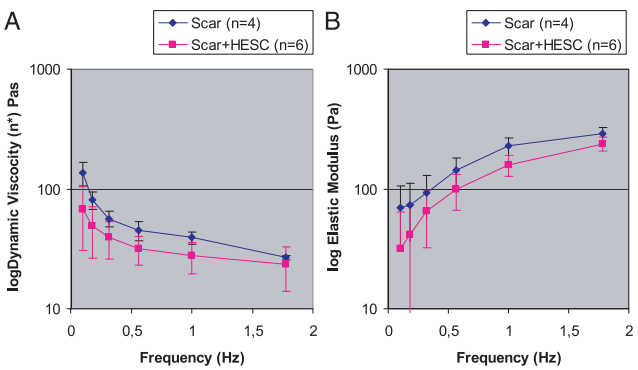

Fig. 3. Result of the reologic measurements showing the dynamic viscosity plotted against frequency (A) and elastic modulus plotted against frequency (B). Within bracket is the standard error of means.

the opportunity to develop into maturer or even adult tissues, are needed before we can understand the risks when using these cells for clinical purposes. A long-term study is ongoing and is hoped to reveal a possible occurrence of such later effects.

Hematoxylin-eosin staining indicated improved wound healing, in that a lower occurrence of polyp formation was detected in the hESC-treated vocal folds. Furthermore, functional studies measuring the viscosity and elastic modulus of the vocal folds revealed that the scarred and hESC-injected vocal folds also showed a significantly reduced viscosity and less elastic modulus compared to the sham-treated scarred vocal folds.

Scarring and stiffness of vocal folds has previously been attributed to increased collagen content of the lamina propria.[17] Thus, improved wound healing is possibly accompanied by reduced levels of collagen. In a study using human adult MSC in the regeneration of scarred rabbit vocal folds, we found that MSC injection significantly reduced the content of collagen type 1, possibly contributing to a parallel observed functional improvement in the MSC-injected vocal folds.[9] In contrast, our present study using hESC indicated no significant differences in collagen type 1 levels when comparing the stem cell-injected vocal folds and the untreated scarred folds. The exception was one hESC-injected fold with high collagen type 1 content and polyp formation, as seen with hematoxylin-eosin (H&E) staining. This indicates that the functional improvement observed was not due to levels of collagen type 1, but rather depended on other factors. Furthermore, it cannot be excluded that the collagen type 1 found in the hESC-treated folds influences the differentiation of the transplanted hESC with a positive functional result. Recently, Leor et al. showed that fetal human umbilical cord blood-derived stem cells could prevent scar thinning in athymic nude rats following induced myocardial infarction.[18] A strong correlation between scarring and collagen levels suggests that early types of human stem cells might have a collagen-increasing effect in wound-healing. Sato et al. showed that enhanced synthesis of collagen in in vitro cultures of murine embryonic stem cells stimulated differentiation into tissues of mesodermal origin, including cartilage and muscle.[19] This leads to the suggestion that collagen production in scar formation might stimulate the transplanted cells to differentiate into the mesodermal lineage, and with possible species differences in mind, this could be an explanation for the rapid differentiation and development of cartilage and muscle in our study. Interestingly, formation of cartilage per se is not likely to contribute to the improved viscoelasticity, rather the opposite, which is why the reason for this effect must be further explored. In addition to collagen type 1, other factors and molecules may be important to vocal fold function in the treatment of scarring, such as the content of elastin, fibromodulin, fibronectin, and procollagen/collagen type 3. The relative proportions of these molecules in the extracellular matrix are likely to be important, and the functional improvement is possibly due to changed connections caused by changes in the matrix protein levels, rather than the reformation of tissue.

The data presented do not exclude a possibility that the immunosupression per se was responsible for the effects observed. However, in the same model, we have in other studies not seen any positive effects on wound healing by giving only immunosuppression to the vocal fold scarred animals (Svensson et al., 2007, manuscript in preparation). In this context it is interesting to note that H&E-stainings revealed signs of immune reactions with host lymphocyte infiltration, despite immunosupression. Thus, the immunosuppressant regimen used did not entirely block the immune effects.

From a clinical point of view, there is still much to learn before hESC derivatives can be used in cell therapy and transplantation. Our previous results using MSC in the same model reveals, however, that this might be suitable for clinical purposes (Hertegård et al., 2006). Even though the MSC do not show the same ability to tissue regeneration, they have a significant functional effect without apparent risks for anaplasia.

CONCLUSION

The data presented indicate that following in vivo injection into adult rabbit vocal folds, hESC not only have the ability for survival and engraftment (5.1%), but also show correlation to local differentiation signals leading to regional specific formation of cartilage, muscle, and epithelium. The enhanced tissue repair was paralleled with a functional regeneration, measured as improved viscoelastic properties, of the vocal folds after scarring. Within the short time frame of the study, no histologic anaplasia was observed. Although the findings are encouraging and verify the potential of functional replacement therapy for scarred vocal folds, the risk for adverse formation of unwanted tissue, indicated by findings in 1 of 22 vocal folds, needs to be further investigated. Ongoing studies will also reveal the long-term persistence of cells and function.

Acknowledgments

In Memoriam: Professor Åke Dahlqvist from Umeå University Hospital, one of the initiators of this project, died in the tsunami catastrophe in Thailand, December 2004. The authors thank Anna Tolf, Department of Pathology, Karolinska University Hospital Huddinge, for help with interpretation of histologic findings.

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III

The Laryngoscope

Injection of Human Mesenchymal Stem Cells Improves Healing of Scarred Vocal Folds: Analysis Using a Xenograft Model

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Objectives/Hypothesis: The aims were to analyze if improved histological and viscoelastic properties seen after injection of human mesenchymal stem cells (hMSCs) in scarred vocal folds (VFs) of rabbits are sustainable and if the injected hMSCs survive 3 months in the VFs.

Study Design: Experimental xenograft model.

Methods: Eighteen VFs of 11 New Zealand white rabbits were scarred by a bilateral localized resection. After 3 months the animals were sacrificed. Twelve VFs were dissected and stained for histology, lamina propria thickness, and relative collagen type I analyses. The hMSCs survival was analyzed using a human DNA-specific reference probe, that is, fluorescence in situ hybridization staining. Viscoelasticity, measured as the dynamic viscosity and elastic modulus, was analyzed in a parallel-plate rheometer for 10 VFs.

Results: The dynamic viscosity and elastic modulus of hMSC-treated VFs were similar to that of normal controls and significantly improved compared to untreated controls ($P < .05$). A reduction in lamina propria thickness and relative collagen type 1 content were also shown for the hMSC-treated VFs compared to the untreated VFs ($P < .05$). The histological pictures corresponded well to the viscoelastic results. No hMSCs survived.

Conclusions: Human mesenchymal stem cells injected into a scarred vocal fold of rabbit enhance healing of the vocal fold with reduced lamina propria thickness and collagen type I content and restore the viscoelastic function.

Key Words: Vocal fold, scarring, viscoelasticity, mesenchymal stem cells.

Level of Evidence: 5

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INTRODUCTION

Scarred vocal folds (VFs) cause severe voice problems.[1] Different etiologies, for example, trauma, surgical defects, and postradiotherapy, are all associated with stiffness of the lamina propria resulting in disturbed viscoelastic properties in the VF.[2] The patient becomes breathy or aphonic, and the phonation threshold pressure that corresponds to ease of phonation is elevated.[2] The treatment is usually difficult, and besides voice therapy it may include injections for augmentation of the VF. Presently, there is no effective method to prevent or heal VF scars or defects. Different substances have been used to augment scar defects in VFs, such as human/bovine collagen,[3,4] autologous fat,[5,6] hyaluronan,[2,7] and autologous implantation of fascia.[8] Tissue engineering approaches, such as injection of hepatocyte growth factor[9] and autologous fibroblasts,[10] have shown improved viscoelastic properties/vibratory characteristics in scarred VFs. Kanemaru et al. showed that injected autologous mesenchymal stem cells in scarred canine and rat VFs healed the VFs well after 2 months macroscopically, and that the stem cells differentiated to epithelial and muscle cells.[11,12]

Human mesenchymal stem cells (hMSCs) have low immunogenicity and persist long term after injection in utero to fetal sheep and in the brain of albino rats.[13,14] Vocal folds represent a relatively closed, poorly vascularized, compartment and might therefore be an immune-

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privileged site that allows engraftment of xenogeneic cells.[15]

In a previous xenograft study with a duration of 1 month, we found a survival rate, engraftment, of 0.18% for hMSCs injected into scarred rabbit VFs. The functional viscoelastic properties, measured as the dynamic viscosity and the elastic modulus of the VFs treated with hMSCs, were significantly improved compared with untreated scarred VFs. The relative amount of collagen type 1 as a measurement of lamina propria scarring was reduced from 2.9% to 1.5% for the hMSC-treated scarred VFs.[16]

Using a xenograft model, the aims of this study were to analyze if the results of improved histological and viscoelastic properties in scarred VFs of rabbits, shown after 1 month, are sustainable over a longer time and if the injected hMSCs survive 3 months in the VFs.

MATERIALS AND METHODS

The principle study design was used previously by several investigators.[2,7,9,11,16] Rabbits were chosen for their relatively small size, but having VFs large enough for the surgical procedures, and for providing a three-layered VF structure, allowing measurements of the lamina propria. American and Swedish principles of laboratory animal care were followed. The experiment was approved by the local ethic committee of the Karolinska Institute. Eleven female New Zealand white rabbits (body weight, 3.0 kg to 4.0 kg) were used in the experiment. Data for five normal VFs were collected from a data bank from earlier experiments.

Vocal Fold Scarring

After premedication with glycopyrrolate (0.1 mg/kg, subcutaneous) and Hypnorm (fentanyl citrate 0.3 mg/mL, mixed with fluanisone 10 mg/mL, 0.3 mL/kg intramuscular; Janssen Pharmaceutica, Beerse, Belgium), all animals were anesthetized with diazepam (2 mg/kg intravenous).

The laryngeal structures and the mobility of the cricoarytenoid joints were found normal at examination by means of a modified 4.0-mm pediatric laryngoscope (model 8576E; Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7-mm rigid endoscope (model 7218A; Karl Storz Endoscope). A digital video recorded on a computer was made of the VFs before and after the scarification procedure (Richard Wolf video camera No. 5512; Richard Wolf Medical Instruments Corp., Vernon Hills, IL; and a Canopus ADVC100 digital video converter, Canopus Electronics Ltd. Hong Kong). The scarring procedure was performed with a 1.5-mm microcup forceps (MicroFrance Medtronic, Düsseldorf, Germany) excising the mucosa and the superficial layer of the thyroarytenoid muscle.

Eighteen VFs were scarred, and the remaining four VFs were left as normal controls together with five VFs from the data bank (n = 22 + 5).

Mesenchymal Stem Cell Preparation and Characterization

The hMSCs were isolated and expanded from bone marrow (BM) taken from the iliac crest of healthy volunteers as previously described.[16,17] Heparinized BM was mixed with a double volume of phosphate buffered saline (PBS), centrifuged at 900*g*, resuspended and layered over a Percoll gradient (1.073 g/mL; Sigma-Aldrich, St. Louis, MO).The mononuclear cells were collected from the interface, washed, and resuspended in hMSC medium consisting of Dulbecco's Modified Eagle's Medium-low glucose (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Life Technologies). The serum lot was selected on the basis of optimal cell growth and differentiation. The cells were plated in culture flasks (Becton Dickinson Biosciences, Bedford, MA) at a density of 160,000 cells/cm^2. Nonadherent cells were removed after 48 to 72 hours, and the adherent cells were cultured. When >70% confluence was reached, the cells were detached by trypsin and ethylenediaminetetraacetic acid (GibcoBRL, Grand Island, NY) and replated at a density of 4,000 cells/cm^2. The cells were harvested in passage five and classified as mesenchymal stem cells (MSCs) based on their ability to differentiate into fat, bone, and cartilage and by flow cytometric analyses (positive for CD73, CD90, CD105, and CD166; negative for CD14, CD31, CD34, CD45 and CD80).

Vocal Fold hMSC Injections

The hMSCs were centrifuged and washed from the growth medium in NaCl and suspended in saline. The solution was aspirated to fill a Medtronic Xomed laryngeal injector with a 27-gauge needle with a syringe of 1 mL. Injections were made under video monitoring directly after the scarification procedure into the lamina propria and/or the superficial part of the thyroarytenoid muscle of the VFs.[16] Ten out of the 18 scarred VFs were injected with hMSCs in 0.1 mL saline each. Each VF was injected with about 80,000 to 100,000 hMSCs. Eight scarred VFs were injected with only 0.1 mL saline, and four VFs were injected with 0.1 mL saline without scarring (controls, together with four VFs from the databank, identically treated).

The animals that received hMSCs were treated with immunosuppressant (tacrolimus, 0.05 mg/kg, body weight subcutaneous) every second day. The dose was based on the recommended dose/kg from the manufacturer and on our previous experiments in rabbits.[16]

Dissection

After 3 months the rabbits were sacrificed with an overdose of sodium pentobarbital. Each larynx was dissected out and divided in the posterior midline. Twelve hemilarynges were placed in 4% formaldehyde for histological and antibody analysis. Ten hemilarynges were fresh frozen at −70°C for viscoelastic analyses. All four normal control samples were used in the viscoelastic analyses and the five normal controls from the databank in the histological analyses.

Histological Measurements

After fixation in 4% formaldehyde and 70% ethanol, the VFs removed from the larynges were further processed, dehydrated, and finally embedded in paraffin wax and cut into 5-μm-thick horizontal sections covering the whole thickness of each VF. Staining was made with hematoxylin and eosin for histological analyses. Image analyses were made at 10× or 20× magnification after digitization of the microscopic images. The slides were blindly analyzed at the Department of Pathology, Karolinska University Hospital Huddinge, Sweden.

Immunohistochemistry for Collagen Type 1 Staining

Staining was performed as previously described.[16,18] Briefly, slides were deparaffinized in xylene, rehydrated in alcohol, and

blocked in PBS containing 3% bovine serum albumin. Slides were incubated with a primary antibody (antibody 6308; Abcam, Cambridge, UK), followed by incubation with a secondary antibody (nr.A21127; Jackson ImmunoResearch Labs Inc., West Grove, PA). Sections were rehydrated in ethanol and xylene and mounted with Vectashield containing DAPI (Vector Labs Inc., Burlingame, CA). The relative contents of collagen type 1 in the VFs were measured from the digitized stains after a color-filtering and normalization process with Photoshop version 8.0 (Adobe Inc., San Jose, CA) and custom-made software (Hans Larsson, Karolinska Institute, Department of Logopedics and Phoniatrics).

Lamina Propria Thickness

Measurements of the lamina propria (LP) thickness were made on the digitized hematoxylin and eosin image of each sample using ImagePro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD). The thickest parts of the LP were measured at three spots for each VF. If a tendency toward polyp formation was seen, six measure points including the polyp were used. Each single value was then used in the statistic evaluation. The LP of two of the untreated VFs (scar + NaCl) were crosscut in the preparation processes and were left out.

Verhoeff Staining

Verhoeff staining was performed to detect elastin. The slides were deparaffinized and rehydrated as above and incubated in Verhoeff solution, containing hematoxylin, ferric chloride, and potassium iodine, for 1 hour at room temperature. The slides were then differentiated in 2% aqueous ferric chloride for 2 minutes and subsequently treated with 5% sodium thiosulphate for 1 minute and counterstained with Van Gieson solution (HT254, Sigma-Aldrich). Finally, the slides were dehydrated, mounted, and digitized as described for the collagen staining.

Alcian Blue Staining

Alcian blue staining was performed to detect mucopolysaccharides and glycosaminoglycans, that is, hyaluronic acid. The slides were deparaffinized and rehydrated in a series of alcohol and incubated in alcian blue stain (alcian blue kit #SS012, BioGenex, San Ramon, CA), for 1 hour at room temperature and counterstained with nuclear fast red solution for 5 minutes. Finally, the slides were dehydrated, mounted, and digitized as described for the collagen staining.

Fluorescence In Situ Hybridization—FISH Analysis—for Persistence of Transplanted hMSCs

Detection of human cells in the VFs were done with a human DNA-specific reference probe, similar to an antibody, linked to a fluorescent molecule, (i.e., FISH analysis). The FISH analysis was performed as previously described.[16,18] Briefly, slides were deparaffinized in xylene and rehydrated in alcohol, followed by pretreatment with pepsin and hybridization over night at 38°C with the human specific fluorescent probe (Spectrum Red human genomic DNA; Vysis Inc., Burlingcame, CA). The detected human cells were counted.

Viscoelastic Measurements

Parallel-plate rheometry. The linear viscoelastic shear properties of VF tissue have been studied by several researchers.[19,20] The parallel-plate rheometer in this experiment produces sinusoidal shear small amplitude oscillations at increasing frequency (from 0.01–15 Hz). We used an AR 2000 Rheometer (TA Instruments, New Castle, DE) with a stationary lower plate (8-mm diameter) separated by about 0.5 mm from a rotating upper plate. Tissue samples from the 10 fresh-frozen VFs were thawed in room temperature, dissected, and analyzed at 37°C in the parallel-plate rheometer (three scarred injected with hMSC, three scarred injected with saline, and four untreated). The samples included lamina propria and the superficial part of the thyroarytenoid muscle. The tissue was kept moistened with saline during the measurements. All rheometric measurements were performed in the linear region with constant strain level transferred from the sample to the upper plate where it was measured with a linear variable displacement transducer. In this experiment the response and reproducibility was stable up to 2 to 3 Hz. For higher frequencies the results were not stable, probably due to inertia of the measurement system because the tissue samples were not geometrically perfectly flat and did not completely fill out the 8-mm plate space. The dynamic viscosity (η', Pas) and elastic modulus (G′, Pa) were derived as a function of frequency. Dynamic viscosity is a measure of a material's resistance to shear flow. The elastic (storage) modulus (G′) represents a measure of a materials stiffness in shear. As mentioned in this experiment, the gap between the plates was not completely filled with tissue. Thus, the absolute level of η' and G′ may not be accurate. However, the same dissection procedure and amount of tissue was used for all samples, which allows for comparison between the different groups.

Statistics

Nonparametric comparisons were made using the Mann-Whitney *U* test. For the histological measurements, each single value was included when differences between the groups were calculated. Significance was considered when $P < .05$.

RESULTS

Viscoelastic Analyses

Dynamic Viscosity (η', Pas). Scarring significantly increased the dynamic viscosity ($P = .012$) (Fig. 1A). Treatment with hMSCs significantly decreased the dynamic viscosity compared with untreated controls ($P < .05$) and was not significantly different from unscarred (normal) controls.

Elastic Modulus (G′, Pa). Scarring also significantly increased the elastic modulus indicating stiffer folds ($P < .05$) (Fig. 1B). Treatment with hMSCs significantly decreased the elastic modulus in comparison with the untreated controls ($P < .05$). No significant difference was shown for the hMSC-treated VFs compared with the unscarred (normal) VFs.

FISH Analysis for Persistence of Transplanted hMSCs. About 10,000 cells per VF were counted. No hMSCs were detected in any of the treated VFs.

Histological Analyses

Collagen type 1 staining. A significant reduction in collagen type I content was shown for the hMSC-treated VFs compared with the untreated VFs ($P = .034$). No significant difference in relative percentage of

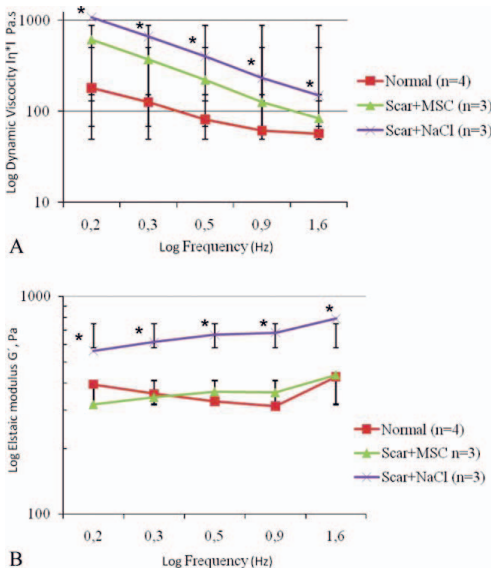

Fig. 1. Rheological data showing (A) dynamic viscocity and (B) elastic modulus, plotted against frequency. Both were significantly reduced in the vocal folds treated with human mesenchymal stem cells (MSCs) compared to untreated folds (scar + NaCl) ($P < .05$). Properties of MSC-treated vocal folds did not differ from normal folds. *$P < .05$; mean value, ±2 standard deviation.

collagen type I content was shown for hMSC-treated VFs compared to the normal VFs. Normal VFs showed significantly lower collagen percentage compared with untreated VFs ($P = .037$) (Fig. 2A).

Lamina propria thickness. A significant reduction in LP thickness was shown between hMSC-treated VFs and untreated VFs ($P = .006$). No significant difference was shown in LP thickness between hMSC-treated VFs and normal VFs ($P = .051$). A significant difference was shown between normal VFs and untreated VFs ($P < .001$) (Fig. 2B).

Hematoxylin and eosin staining. The VFs were characterized in four categories depending on grade of scarring (fibrosis). Grade A showed none or minimal signs of fibrosis. Grade B showed a focal or noncompact fibrosis in the LP or superficial vocal muscle. Grade C showed a more compact fibrosis in the LP and superficial muscle, and grade D a compact fibrosis in LP and superficial muscle as well as fibrosis in the deeper part of the vocal muscle (Fig. 3).[21]

All but one of the untreated VFs (scar + NaCl) were placed in group C or D (n = 5). Two of the hMSC-treated VFs (n = 7) were placed in group C and none in group D. When the A and B groups were compared with the C and D groups, although a clear tendency but not significant, the hMSC-treated VFs were placed in the A and B group and the untreated VFs in the C and D group.

Verhoeff staining. The differences in elastin content between the groups were small. No statistics were obtainable. The stainings were judged visually and blindly for each sample. The VFs treated with hMSCs showed a normal distribution or a tendency to slightly increased elastin content. When polyp formation occurred elastin seemed to be reduced. In the untreated VFs elastin tended to be reduced.

Alcian blue staining. The VFs were analyzed as the Verhoeff stainings. The alcian blue-stained hyaluronic acid was distributed in a very scarce patchy manor in the LP and the superficial muscle of the normal VFs. The VFs treated with hMSCs showed a tendency of diminished amount distributed mainly anteriorly and near the cartilage. Only one of the hMSC-treated VFs showed a normal amount and distribution. For the untreated VFs, samples of both increased and of total lack of alcian blue were found.

DISCUSSION

The results confirm our earlier 1-month study that hMSCs have the potential to significantly improve histological and viscoelastic properties of scarred VFs. Moreover, the improved healing is sustainable over a longer period.

The hMSCs did not survive 3 months in the VFs of rabbits. This is to be interpreted in a positive way, as it means that no hMSCs linger in the VFs after the healing process is completed. If transferable to a clinical setting this is advantageous, as it is preferable that no potent cells remain in the tissue after healing. However, this needs to be shown also in an autologous setting.

The hMSCs are immunosuppressive in vitro and reduce lymphocyte proliferation and formation of cytotoxic T cells.[22] MSCs have also been found to enhance

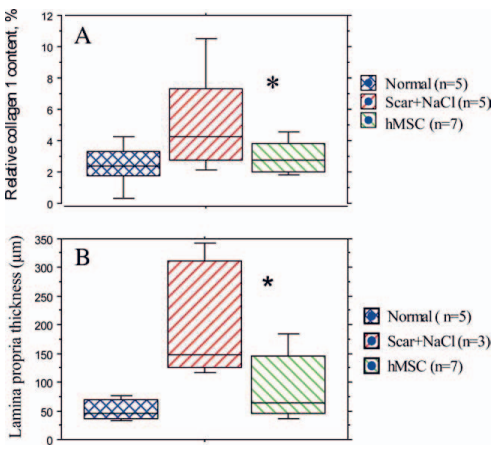

Fig. 2. (A) Relative collagen type 1 (%) and (B) lamina propria thickness (μm) in normal vocal folds compared to untreated scarred folds and folds treated with human mesenchymal stem cells (hMSCs). Within boxes is median, 25th and 75th percentiles; outside of boxes are 10th and 90th percentiles. *$P < .05$.

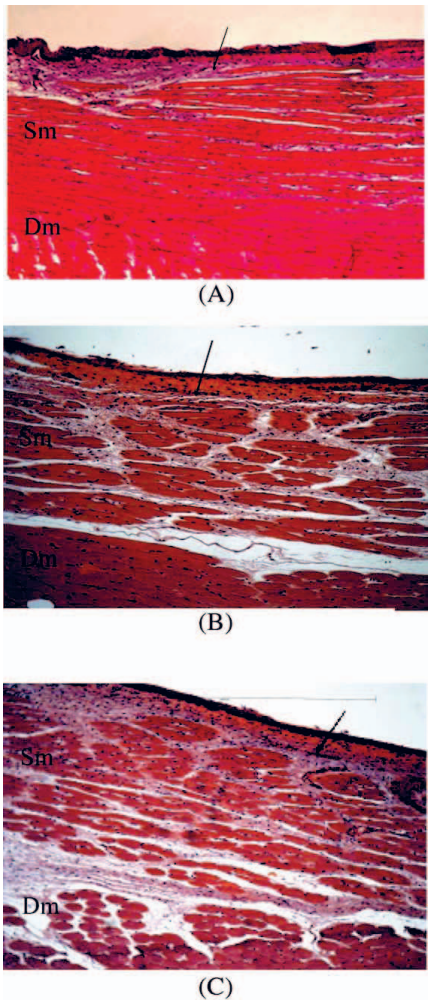

Fig. 3. Representative hematoxylin and eosin stainings of (A) normal vocal fold (VF), showing none, or to the left only minimal loose connective tissue superficially under the lamina propria (LP) (group a). (B) Scarred VF treated with human mesenchymal stem cells showing loose connective tissue that tends to split up the superficial muscle (Sm) bundles, but deep muscle is unaffected (group b). (C) Untreated VF (scar + NaCl), showing compact connective tissue/fibrosis in deep LP expanding into Sm and down into the deep muscle (Dm) (group d). The groups a, b, and d refer to the grade of fibrosis classification described in the text. Arrows mark the border between LP and Sm (10× magnification).

secretion of cytokines and chemokines.[23] Our findings corroborate with the results of other investigators who have found improved wound healing after treatment with MSCs in various skin wounds.[24–26] A reduced inflammatory response induced by the hMSCs appears to be essential in explaining the improved healing measured as a reduction of the collagen 1 content and the LP thickness. Also, there is no significant difference between hMSC-treated VFs compared to normals in the LP thickness or collagen type 1 content; there are differences in the histological picture when individual samples are compared. A tendency to thicker epithelium and unevenly expressed fibroses in the LP and superficial vocal muscle were seen in most of the hMSC-treated samples.

Hyaluronic acid (HA) has been shown to play an important role in the biochemics and function of the VFs.[19] In this study we found only a tendency of reduced alcian blue as a measurement of HA in the hMSC-treated VFs compared to normals. The alcian blue was not patchy scattered as in the normals but localized anteriorly and near the cartilage. It has been suggested that HA is produced by stellate-shaped fibroblasts in the macula flava regions, anteriorly and near the cartilage.[27] The alcian blue stain localization in the hMSC-treated VFs might indicate a HA depletion in the scarred center with increased production in the flava regions (Fig. 4).

The results can be seen from the perspective that there is no ideal animal model to represent the human VFs. However, higher mammals, for example, rabbits and canine, seem to have a rather similar VF healing capacity and pattern of scar formation compared to humans.[28–30] Therefore, it seems reasonable to assume that the histological and viscoelastic findings are transferable to the human VFs.

In the study, an immunosuppressant was used to reduce the host versus graft reaction. This may have influenced the inflammatory reaction and also affected the hMSCs. In a study comparing the healing process with and without an immunosuppressant, we did not find any improved healing with an immunosuppressant alone (unpublished data). If the immunosuppressant has had any effect on the hMSCs, it is reasonable to believe it has been negative. If so, the hMSCs have the potential to enhance the results in an autologous environment.

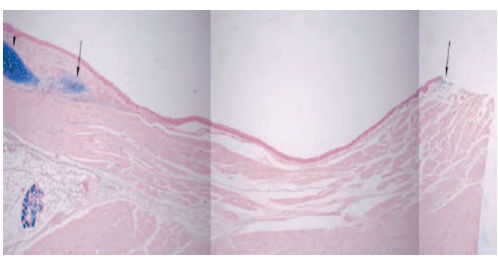

Fig. 4. Alacian blue staining (AC) of the whole length of a human mesenchymal stem cell treated vocal fold (VF). The AC shows the hyaluronic acid distribution at the anterior and posterior part of the VF (faint). Arrows mark macula flava posterior and anterior. Arrowhead marks the cartilage of the vocal process (2× magnifications, montage.)

CONCLUSION

Human mesenchymal stem cells injected into the scarred vocal folds of rabbits enhance healing of the vocal folds with reduced lamina propria thickness and relative collagen type 1 content, rendering restored viscoelastic function to the vocal folds. Human mesenchymal stem cells do not seem to survive 3 months in the vocal folds of rabbits.

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IV

The Laryngoscope

Injection of Human Mesenchymal Stem Cells Improves Healing of Vocal Folds after Scar Excision—A Xenograft Analysis

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Objectives: Using a xenograft model the aim was to analyze if injection of human mesenchymal stem cells (hMSC) into the rabbit vocal fold (VF), after excision of an established scar, can improve the functional healing of the VF.

Study Design: Prospective design with an experimental xenograft model.

Methods: The VFs of 12 New Zealand rabbits were injured by a bilateral localized resection. After 9 weeks the scar after the resection was excised and hMSC were injected into the VFs. After another 10 weeks 10 VFs were dissected and stained for histology. Lamina propria thickness and relative content of collagen type I were measured. Viscoelasticity of 14 VFs at phonatory frequencies was quantified by a simple-shear rheometer. The hMSC survival was determined using a human DNA specific reference probe, that is, FISH analysis.

Results: The viscoelastic measurements, that is, dynamic viscosity and elastic shear modulus for the hMSC-treated VFs, were found to be similar to those of normal controls and were significantly lower than those of untreated controls ($P < .05$). A significant reduction in lamina propria thickness was also shown for the hMSC treated VFs compared with the untreated VFs ($P < .05$). This histologic finding corresponded with the viscoelastic results. No hMSC survived 10 weeks after the injection.

Conclusions: Human mesenchymal stem cells injected into the rabbit VF following the excision of a chronic scar, were found to enhance the functional healing of the VF with reduced lamina propria thickness and restored viscoelastic shear properties.

Key Words: Vocal fold, scar, viscoelasticity, mesenchymal, stem cells.

Level of Evidence: 5

***Laryngoscope,* 121:2185–2190, 2011**

INTRODUCTION

Tissue defects in the vocal fold (VF) caused by trauma, surgical procedures, or postradiotherapy, often heal with scar formation. The scar tissue causes stiffness of the lamina propria rendering disturbed viscoelastic properties in the VF.[1] A scarred VF causes severe voice problems.[2] Treatment is difficult, and besides voice therapy, injections are used for augmentation of the VF scar defects. Different injectable substances have been used, for example, human/bovine collagen,[3,4] autologous fat,[5,6] hyaluronan,[2,7] and autologous implantation of fascia.[8] Tissue engineering approaches, such as injection of hepatocyte growth factor[9] and autologous fibroblasts,[10] have shown improved viscoelastic properties/vibratory characteristics in scarred VFs, but presently there are no effective methods to prevent scarring of VF defects or to heal VF scars.

Human mesenchymal stem cells (hMSCs) have a multilineage potential to develop into myelosupportive tissue, muscle, cartilage, and bone.[11] Autologous MSCs injected in scarred canine and rat VFs have been shown to differentiate into epithelial and muscle cells and macroscopically heal the VFs well.[12,13] hMSCs have also been shown to have immunomodulative properties reducing the inflammatory response of lymphocytes and T cells.[14–16]

In two previous xenograft studies using hMSCs injected in injured VFs of rabbits, we found a survival rate of the hMSCs, engraftment, of 0.18% after 1 month and no survival of hMSCs after 3 months. Both studies also showed that the VFs treated with hMSCs gained significantly improved viscoelastic function, measured as dynamic viscosity and elastic modulus compared with

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untreated VFs. The relative amount of collagen type I and lamina propria (Lp) thickness as a measurement of Lp scarring were significantly reduced for the hMSC-treated VFs compared to the untreated VFs and the improvements were sustainable over 3 months.[17,18]

Using a xenograft rabbit model, the aim of this study was to analyze if the results in our previous studies showing improved healing of injured VFs after an injection of hMSCs into the injured VFs are transferable to a clinical-like situation of excising an established scar followed by an immediate injection of hMSCs into the scar-excised VF.[19]

MATERIALS AND METHODS

The principal study design has been used by several investigators.[2,7,9,12,17]

United States and Swedish principles and protocols of laboratory animal care were followed. The experiment was approved by the local ethics committee of the Karolinska Institute, Sweden. Twelve female New Zealand white rabbits (body weight [bw] 3.0–4.0 kg) were used in the experiment. Additional data for five normal VFs were collected from a data bank from earlier experiments and were used in the histologic analyses.

VF Scarring and Scar Excision

Ten animals were anesthetized for each procedure. Two animals remained untreated and were used as normal controls in the rheology measurements.

After premedication with glycopyrolate (0.1 mg/kg s.c.) and Hypnorm® (fentanylcitrate 0.3 mg/mL mixed with fluanizonum 10 mg/mL, 0.3 mL/kg i.m., Janssen Pharmaceutica, Beerse, Belgium) the animals were anesthetized with diazepam (2 mg/kg i.v.) The laryngeal structures and the mobility of the cricoarytenoid joints were found normal at examination by means of a modified 4.0-mm pediatric laryngoscope (model 8576E, Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7-mm rigid endoscope (model 7218A). A digital video recorded on a computer was made of the VFs before and after the scarification and the scar excision procedure (Richard Wolf video camera No 5512 and a Canopus ADVC100 digital video converter, Reading, UK). The scarring procedure was performed with a 1.5-mm microcup forceps (MicroFrance Medtronic, Düsseldorf Germany) excising the mucosa (Lp) and the superficial layer of the thyroarytenoid muscle. After 9 weeks a scar excision procedure was performed with a 2-mm microcup forceps (MicroFrance Medtronic, Düsseldorf, Germany) excising all visible scarred tissue. All 12 animals were sacrificed after another 10 weeks. Twenty VFs were operated upon and the remaining four VFs were left as normal controls together with the five VFs from the data bank, that is, total $n = 24 + 5$.

hMSC Ppreparation and Characterization

hMSCs were isolated and expanded from bone marrow (BM) taken from the iliac crest of healthy volunteers as previously described.[16,17] Heparinized BM was mixed with a double volume of phosphate-buffered saline (PBS), centrifuged at 900 $\times$ *g*, resuspended, and layered over a Percoll gradient (1.073 g/mL; Sigma-Aldrich, St. Louis, MO). The mononuclear cells were collected from the interface, washed, and resuspended in HMSC medium consisting of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic–antimycotic solution (Life Technologies). The serum lot was selected on the basis of optimal cell growth and differentiation. The cells were plated in culture flasks (Becton Dickinson Biosciences, Bedford, MA) at a density of 160,000 cells/cm^2. Nonadherent cells were removed after 48 to 72 hours and the adherent cells were cultured. When >70% confluence was reached, the cells were detached by trypsin and ethylenediamine- tetraacetic (EDTA) (GibcoBRL, Grand Island, NY) and replated at a density of 4,000 cells/cm^2. The cells were harvested in passage five and classified as MSCs based on their ability to differentiate into fat, bone, and cartilage and by flow cytometric analyses (positive to CD73, CD90, CD105, and CD166, negative to CD14, CD31, CD34, CD45, and CD80).

VF hMSC Injections

The hMSCs were centrifuged and washed from the growth medium in NaCl and suspended in saline. The solution was aspirated to fill a Medtronic Xomed laryngeal injector with a 27-gauge needle with a syringe of 1 mL. Injections were made under video monitoring directly after the scar excision procedure into the Lp and/or the superficial part of the thyroarytenoid muscle of the VF.[17,18] The correct injection site was stated by observed bulging of the VF corresponding to the injected volume. Fourteen out of the 20 scarred VF were injected with hMSC in 0.1 mL saline each (seven for histology and seven for rheology measurements). Each VF was injected with about 80,000–100,000 hMSCs. Six scarred VFs were injected with only 0.1 mL saline (3 for histology and 3 for rheology measurements). Four VFs were injected with 0.1 mL saline without scarring (normal controls for rheology measurements). Additionally, five earlier identically treated VFs were taken from a data bank and used as normal controls in the histologic measurements.

The animals that received hMSCs were treated with immunosuppressant (Tacrolimus, TC, 0.05 mg/kg bw s.c.) every second day. The dose was based on the recommended dose/kg from the manufacturer and our previous experiments in rabbits.[17]

Due to an expected higher vulnerability in the immunosuppressed animals, that is, the hMSC-treated groups, they included more samples compared with the untreated and normal groups. The aim was to maintain adequate sample sizes in the immunosuppressed hMSC-treated groups. Only two samples were lost, both in the Lp thickness group. All other groups remained unaffected.

Sample Procurement

After 19 weeks the rabbits were sacrificed with an overdose of pentobarbital sodium. Each larynx was dissected out and divided in the posterior midline. Ten hemilarynges were placed in 4% formaldehyde for histologic and antibody analyses. Fourteen hemilarynges were quick frozen at −70°C for viscoelastic analyses.

Histologic Measurements

After fixation in 4% formaldehyde and 70% ethanol the VFs removed from the larynges were dehydrated and finally embedded in paraffin wax. Each VF was cut into 5-μm-thick horizontal sections covering the whole thickness of the VF. On average, 30 sections per VF were generated. For each staining method two to four slides from different levels of each VF were analyzed. Staining was made with hematoxylin-eosin for histologic analyses. Image analyses were made at 10$\times$ or 20$\times$ magnification after digitization of the microscopic images.

The slides were blindly analyzed at the Department of Pathology, Karolinska University Hospital Huddinge, Sweden. Intrareliability was assessed by blind reexamination of 10% of the slides, randomly chosen. The results were identical.

Immunohistochemistry for Collagen Type I Staining

Staining was performed as previously described.[17,18] Briefly, slides were deparaffinized in xylene, rehydrated in alcohol, and blocked in PBS containing 3% bovine serum albumin (BSA). Slides were incubated with a primary antibody (antibody 6308, Abcam, Cambridge, UK), followed by incubation with a secondary antibody (no. A21127 Jackson ImmunoResearch labs Inc., West Grove, PA). Sections were rehydrated in ethanol and xylene and mounted with Vectashield containing DAPI (Vector Labs Inc., Burlingame, CA). The relative contents of collagen type I in the VFs were measured from the digitized stains after a color filtering and normalization process with Photoshop (version 8.0) and a custom-made software (Hans Larsson, Karolinska Institute, Departmentof Logopedics and Phoniatrics).

Lp Thickness

Measurements of the Lp thickness were made on the digitized hematoxylin-eosin image of each sample (custom-made software Hans Larsson, Karolinska Institute, software Image Pro Plus® version 3.0 Media Cybernetics). The thickest parts of the Lp were measured at three spots for each VF. If a tendency of polyp formation was seen, six measure points including the polyp were used. All measurement values were then used in the statistical evaluation.

The Lp of two of the hMSC-treated VF were damaged in the preparation processes and were left out in the Lp thickness measurements, resulting in n = 5 for hMSC-treated, n = 3 for untreated (scar + NaCl), and n = 5 for normal VFs.

Verhoeff Staining

Verhoeff staining was performed to detect elastin. The slides were deparaffinized and rehydrated as above and incubated in Verhoeff solution, containing haemotoxylin, ferric chloride, and potassium iodine, for 1 hour at room temperature. Then differentiated in 2% aqueous ferric chloride for 2 minutes and subsequently treated with 5% sodium thiosulphate for 1 minute and counterstained with Van Gieson solution (HT254, Sigma-Aldrich, Inc.). Finally, the slides were dehydrated, mounted, and digitized as described for the collagen staining.

Alcian Blue Staining

Alcian blue staining was performed to detect mucopolysaccharides and glycosaminoglycans, for example, hyaluronic acid. The slides were deparaffinized and rehydrated in series of alcohol and incubated in alcian blue stain (Alcian blue kit, #SS012, BioGenex, CA), for 1 hour at room temperature and counterstained with nuclear fast red solution for 5 minutes. Finally, the slides were dehydrated, mounted, and digitized as described for the collagen staining.

Fluorescence in situ hybridization—FISH analysis—for persistence of transplanted hMSCs.

Detection of human cells in the VFs was done with a human DNA-specific reference probe, similar to an antibody, linked to a fluorescent molecule, that is, FISH analysis.

The FISH analysis was performed as previously described.[17,20] Briefly, slides were deparaffinized in xylene and rehydrated in alcohol, followed by pretreatment with pepsin and hybridization over night at 38°C with the human specific fluorescent probe (Spectrum Red human genomic DNA, Vysis Inc., Burlingame, CA). The detected human cells were counted.

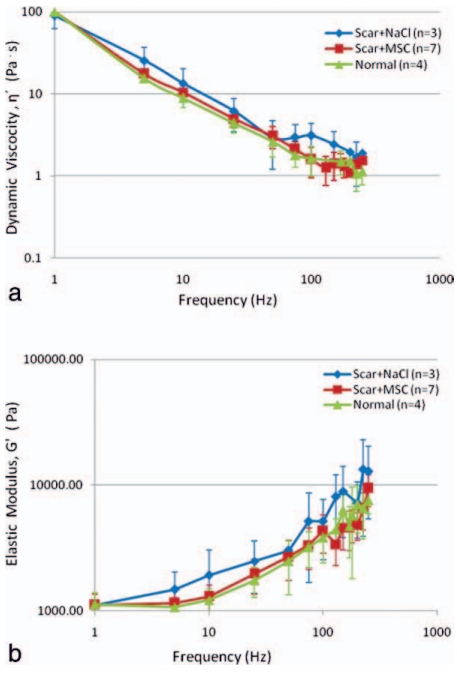

Fig. 1. Rheological data showing (a) dynamic viscocity, (b) elastic modulus, plotted against frequency. Both were significantly reduced in the vocal folds (VFs) treated with human MSC compared to untreated VFs (scar + NaCl) ($P < .05$). Properties of MSC-treated VFs did not differ from normal VFs ($P > .05$). All data points represent mean value ±2 SD.

Viscoelastic Measurements

The linear viscoelastic shear properties of VF tissues have been quantified in previous studies.[21,22] In this study, a controlled-strain, linear, simple-shear rheometer based on the EnduraTEC ElectroForce 3200 mechanical testing system (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN) was used.[21] The rheometer was capable of empirical measurements of viscoelastic shear properties at phonatory frequencies, following validation previously.[21] In the rheometer, a specimen was sandwiched between an upper plate and a lower plate separated by 0.5 to 1.0 mm. A translational displacement of a prescribed amplitude and frequency was applied to the specimen through the upper plate. The shear force resulting from the viscoelastic response of the specimen upon oscillatory shear deformation was detected by a piezoelectric force transducer attached to the lower plate. All rheometric measurements were performed in the linear viscoelastic region (at 1%–2% strain) over the frequency range of 1 to 250 Hz. The elastic shear modulus (G', in Pa) and the dynamic viscosity (η', in Pa · s) were derived as functions of frequency according to the theory of linear viscoelasticity.[21] The elastic (storage) modulus (G') represents a measure of the specimen's stiffness under shear deformation, whereas the dynamic viscosity is a measure of the specimen's resistance to shear flow, or energy dissipation.

Measurements of G' (Pa) and η' (Pa · s) as functions of frequency, f (in Hz) were plotted in log–log scale as shown in Figure 1a and b. Curve-fitting regression was then performed

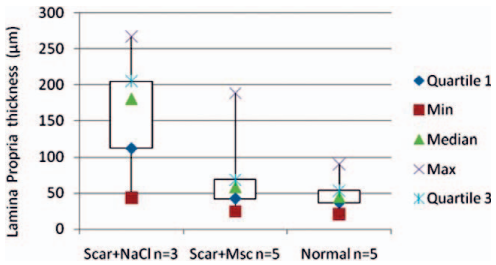

Fig. 2. Lamina propria (Lp) thickness (μm), in untreated scarred (scar + NaCl) vocal folds (VF) compared to folds treated with human MSC (scar + MSC) and normal VFs. A significant reduction in Lp thickness was shown between MSC-treated VFs and untreated VFs ($P < .05$). No significant difference was shown in Lp thickness between MSC-treated VFs and normal VFs.

for each curve to examine the relationships between G' and f and between η' and f. The linear model was used for both G' and η', that is, $\log(G' \text{ or } \eta') = B_0 + B_1 \cdot \log(f)$, where B_0 and B_1 are coefficients of parameterization.[21] The curve-fitting estimations, based on least-squares regression analysis, resulted in highly significant findings using the ANOVA F test in all cases ($P < .001$). The significant values of the F test suggested that the variation explained by the model was not due to chance. Goodness of fit was also estimated by the coefficient of determination, R^2. The R^2 statistic is a measure of the strength of association between the observed and model-predicted values for both $\log(G')$ and $\log(\eta')$. The values of R^2 were high for each regression model indicating goodness of fit ($R^2 > .86$ for $\log[G']$ and $R^2 > .96$ for $\log[\eta']$).

Statistical Analyses

For the histologic measurements each single value was included when differences between the various groups were estimated. Differences between two groups were assessed using Mann–Whitney U test. Calculations whether or not, the dynamic viscosity and the elastic modulus, respectively, differed between normal VFs, hMSCs treated VFs, and untreated scarred controls, were performed with the binomial test. In regression analysis the F test was used. When a direction of the difference between two samples could be predicted as to their means, that is, the mean of a sample 1 was "greater than" or "less than" that of sample 2, the test was one tailed, otherwise it was two tailed. Statistical significance was considered when $P < .05$.

RESULTS

Viscoelastic Analyses

Dynamic viscosity, η' (Pa · s). Scarring significantly increased the tissue dynamic viscosity compared to normal VFs ($P = .01$). Treatment with hMSC significantly decreased the dynamic viscosity compared with the untreated scarred controls (scar + NaCl) ($P = .03$), and hMSC-treated VFs were not significantly different from the unscarred controls, that is, normal VFs ($P = .2$) (Fig. 1a).

Elastic shear modulus, G′ (Pa). Scarring also significantly increased the tissue elastic modulus indicating stiffer VFs, compared to normal VFs ($P = .003$). Treatment with hMSCs significantly decreased the elastic modulus in comparison with the untreated scarred controls (scar + NaCl) ($P < .001$). No significant difference was found between the hMSC-treated VFs and the unscarred controls, that is, normal VFs ($P = .7$) (Fig. 1b).

FISH Analysis for Persistence of Transplanted hMSCs

No hMSCs were detected in any of the treated VFs according to FISH analysis.

Histologic Analyses

Lp thickness. A significant reduction in Lp thickness was shown between hMSC-treated VFs and untreated VFs (scar + NaCl) ($P = .01$). There was no significant difference between hMSC-treated VFs and normal VFs. A significant difference was shown between normal VFs and untreated VFs (scar + NaCl) (P *< .001) (Fig. 2).

Collagen type I staining. No significant reduction in the relative content of collagen type I was found between hMSC-treated VFs and untreated VFs. But neither were there any significant differences between hMSC-treated VFs and normal VFs. Normal VFs showed significantly lower relative collagen type I compared with untreated VFs ($P < .05$).

Hematoxylin-eosin staining. The VFs were characterized into four categories depending on the grade of scarring, that is, fibrosis. Grade A showed no or minimal signs of fibrosis. Grade B showed a focal or noncompact fibrosis in the Lp or superficial vocal muscle. Grade C showed a more compact fibrosis in the Lp and superficial muscle, and Grade D a compact fibrosis in Lp and superficial muscle as well as fibrosis in the deeper part of the vocal muscle[22] (Fig. 3).

The untreated VFs were placed in group C and D, one in group B (n = 3). One of the hMSC (n = 7)-treated VF was placed in group C, none in group D. When the A and B groups were compared with the C and D groups, there was a clear tendency, although not significant, that the hMSC-treated VFs were placed in the A and B groups and the untreated VFs in the C and D groups.

Verhoeff Staining

The stainings were judged visually and blindly for each sample. The differences in elastin content between the groups were minute and without significance. However, the VFs treated with hMSC showed a normal distribution or a tendency to slightly increased elastin content in both the Lp and the superficial muscle. In the untreated VFs, samples of both increased and reduced elastin content were seen.

Alcian Blue Staining

The VFs were analyzed by Alcian blue similar to the Verhoeff stainings. Alacian blue stained hyaluronic acid was distributed in a very scarce patchy manner in the Lp and the superficial muscle of the normal VF. The

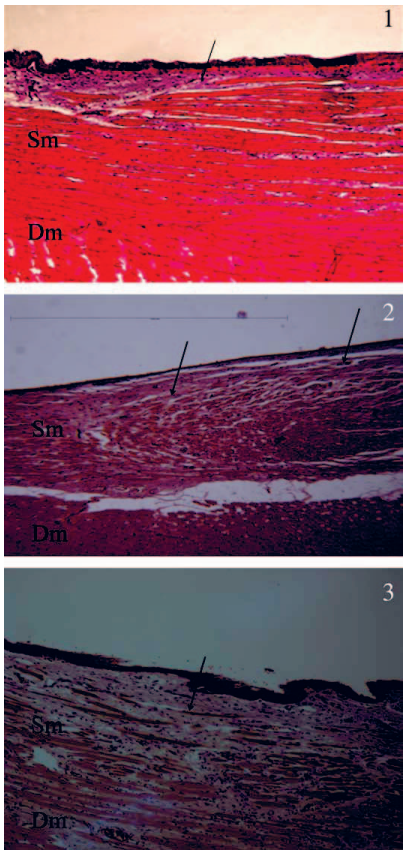

Fig. 3. Representative hematoxylin-eosin stainings of (1) normal vocal fold (VF), showing none, or to the left minimal loose connective tissue superficially under the lamina propria (Lp). Some inflammatory cells in the space of Lp, seen as black dots (group a). (2) Human mesenchymal stem cells-treatedVF, showing loose connective tissue that tends to split up some superficial muscle (Sm) bundles but deep muscle (Dm) is unaffected. Very few inflammatory cells (group b). (3) Untreated VF (scar + NaCl) showing compact connective tissue/fibrosis/ in deep Lp expanding into Sm and down into the Dm. Extensive amount of inflammatory cells deep into Sm and Dm.The Group, a, b, and d, refer to grade of fibrosis classification described in the text. Arrows mark the border between Lp and Sm (10× magnifications, scale bar 1 mm).

VFs treated with hMSC showed a tendency of increased amount and distributed mainly anteriorly and near the cartilage. The untreated VFs showed almost no hyaluronic acid, and if present, it was found at the anterior or posterior end of the VF.

DISCUSSION

The results were consistent with those of our earlier 1-month and 3-month studies, and show that hMSC injected in an injured rabbit VF, improve the functional healing process with significantly improved histologic and viscoelastic properties of the VF. The present study shows that the results are also applicable after a scar excision has been made. The results indicate that the method of excising a scar and thereafter injecting hMSC in the VF in order to restore its viscoelastic function has the potential to work in a clinical setting. Moreover, with the results from the earlier 3-month study, the improved healing can be expected to be sustainable over time.

The hMSC did not survive 10 weeks in the VFs of rabbits. This is interpreted as a positive finding as it means that no hMSC lingered in the VF after the healing process was completed. If transferable to a clinical setting this is advantageous as it is preferable that no potent cells remain in the tissue after healing. This, however, needs to be shown also in an autologous environment.

hMSCs have been shown to be immunosuppressive, reducing lymphocyte proliferation and formation of cytotoxic T cells, as well as enhancing the secretion of

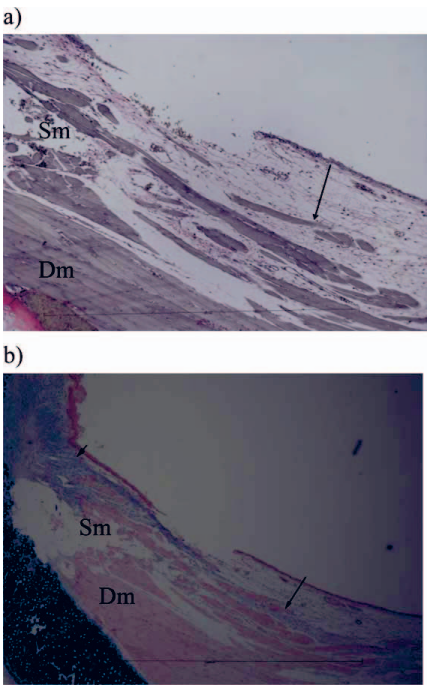

Fig. 4. Human mesenchymal stem cell-treated vocal fold with a damaged part of the epithelium and lamina propria (damaged in the preparation). (a) Verhoeff staining for elastin shows an extremely thick Lp rich in grayish elastin fibers extending down into the superficial muscle (Sm), but no fibrosis is seen. Deep muscle (Dm) is unaffected. (b) The same VF stained with Alcian blue for hyaluronic acid. The bluish hyaluronic acid is richly distributed in the Lp and the superficial part of the Sm. Arrows mark the border between Lp and Sm. Arrowhead marks lamina flava posterior. Dark blue is reference colored cartilage.

chemokines and cytokines.[15,16] A reduced inflammatory response induced by the hMSC seems to have contributed to the improved healing as measured by a significant reduction of Lp thickness indicating reduced fibrosis. A lower grade of fibrosis was also seen in the histologic pictures of the hMSC-treated VFs compared to the untreated controls. The hMSC-treated VFs when histologically classified tended to be placed in the groups with the least fibrosis when a four-grade scale of classification was used.[23] The scarred nontreated controls tended to be placed in the groups with most fibrosis.

One of the two hMSC-treated VFs that was excluded in the Lp thickness measurements due to the Lp being partially split up in the preparation process, showed by Verhoeff staining, extensive elastin production in the Lp and also some in the superficial vocal muscle. In the Alcian blue staining this VF was also found to be rich in hyaluronic acid both in the Lp and the superficial part of the vocal muscle. The VF, however, showed promising viscoelastic shear properties. This might be due to the hMSCs having stimulated the creation of a structured scaffold, in which elastin is a part, in the injured Lp. The hMSC could also have stimulated the production of hyaluronic acid (HA) in this matrix, with HA being beneficial for the healing process and the viscoelastic function of the VF[24,25] (Fig. 4).

In this study immunosuppression was used to reduce the host-versus-graft reaction. This may have influenced the inflammatory reaction and also affected the hMSCs. In a study comparing the healing process with and without the calcineurin inhibitor tacrolimus, we did not find any improved healing with immunosuppressant alone (unpublished data). If the immunosuppressant has had any effect on the hMSC, it is reasonable to believe it has been negative. If so, the hMSCs could have the potential to show even further improved results in an autologous environment.

CONCLUSIONS

hMSCs, when injected into the scarred rabbit VF after a scar excision has been made, were shown to enhance the functional healing of the VF with reduced Lp thickness and restored viscoelastic properties of the VF. The injected hMSCs did not seem to survive for 10 weeks in the rabbit VF.

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Effects of Immunosuppression on Human Mesenchymal Stem Cells in Healing Scarred Rabbit Vocal Folds. Xenograft Analyzes.

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ABSTRACT

Objectives: The aim was to analyze the impact of immunosuppression Tacrolimus (TC) on human mesenchymal stem cells (hMSC) in healing injured rabbit vocal folds (VF).

Study Design: Experimental xenograft model. Prospective design.

Methods: : Twenty-four VFs of New Zealand White rabbits were injured by a 1,5 mm localized resection and injected with hMSCs with and without TC or given TC alone.

After 1 month the VFs were analyzed for lamina propria (Lp) thickness and relative content of collagen type I. The degree of scarring was graded from hematoxylin-eosin stainings**.** The hMSC survival was determined by FISH-analysis.

Result: A significant reduction in lamina propria (Lp) thickness was seen for hMSC treated VFs when compared with treatment with TC alone ($p<.0001$). There was also a significant reduction in Lp thickness for the hMSC treated compared with the hMSC + Tc treated VFs ($p<.014$), as well as for the VFs treated with hMSC+TC compared with only TC treatment ($p<.002$). Furthermore, the content of collagen type I was reduced in the VFs treated with hMSCs when compared with treatment with only TC ($p=.026$). A difference was also found between hMSC treated VFs with and without TC ($p=.015$). No significant difference was seen between the hMSC + TC treated VFs and the VFs treated with only TC.

These observations corresponded well with the histologic picture comparing grade of scaring. The hMSC survival rate, engraftment of 0,09 %, was equal for the VFs with and without TC.

Conclusions: In a xenograft model with injured rabbit vocal folds the immunosuppressant Tacrolimus, shows a significant suppression on the anti-scaring effect of the injected hMSCs. After one month no significant impact on the engraftment of the hMSCs was found.

Short title: Stem cells and immunosuppression in vocal folds

Key words: Mesenchymal, stem cells, scar, vocal folds, immunosuppression, tacrolimus

INTRODUCTION

Surgical procedures or radiation of the vocal folds (VFs) often cause scar formation in the lamina propria (Lp) and in the superficial part of the thyroarytenoid muscle.[1] Scars in the Lp and in the superficial part of the VF render stiffness and disturbed viscoelastic properties to the VF.[2] Scar formation in the VFs causes severe voice problems and is a demanding challenge to treat.[3] Several substances have been used to augment scarred or atrophic VFs, e.g. human/bovine collagen,[4,5] autologous fat,[6,7] hyaluronan,[8,9] autologous implantation of fascia,[10] injection of hepatocyte growth factor[11] or autologous fibroblasts.[12,13,14]

Several of these substances have been shown to improve the vibratory characteristics of scarred VFs, but presently there are no effective methods to prevent or heal VF scars or defects.[15]

In three previous studies using a xenograft model with rabbits, we have shown that injection of human mesenchymal stem cells (hMSC) into injured or scared rabbit VFs heals the VFs with significantly improved viscoelastic properties along with reduced scar formation.[16,17,18] In these studies we used an immunosuppressant, the calcineurin inhibitor tacrolimus (TC) to prevent rejection of the hMSCs. This protocol may have influenced the inflammatory reaction and also affected the hMSCs. Thus it is possible that the immunosuppression per se rather than the stem cells could have contributed to the improved healing.

TC binds to intracellular receptor proteins, specifically to the immunophilin FK 506 binding protein (FKBP). The complex TC-FKBP inhibits transcriptional activity and causes a down regulation of the signal transduction pathways in T cells.[19] This reduces the transcription factors that promote cytokine gene activation.[20] In a rat induced arthritis model, tacrolimus was found to be more effective than methotrexate in reducing elevated levels of inflammatory cytokines, TNF-α, IL-1β, and IL-6.[21] Calcineurin inhibition also reduces activation of enzymes, i.e. cAMP dependent protein kinase, phosphatase 1 and nitric oxide synthetase.

Thus, also cellular effects such as cell degranulation and apoptosis in leukocytes can be affected by TC.[22] TC has also been shown to enhance corticosteroid receptor-mediated gene expressions in vitro.[23]

Using the same xenograft model with rabbits as in our previous studies [16,17,18], the aims of this study were to analyze whether immunosuppression by tacrolimus (TC), per sé affects the survival of human mesenchymal stem cells injected in injured rabbit vocal folds and also if TC affects the scar diminishing effect of the injected hMSCs.

MATERIAL and METHODS

Animal study. The study design is identical with our previous experiments[16,17,18] and has been used by several investigators.[8,9,11,17,18]

U.S. and Swedish principles and protocols of laboratory animal care were followed. The experiment was approved by the local ethics committee of the Karolinska Institute, Sweden. Ten female New Zealand White rabbits (bw 2,7 kg-3.4kg) were used in the experiment. Four VFs from a pilot study with identical procedure were added and additional data for 5 normal VFs were collected from the data bank from earlier experiments.

Vocal fold scarring

After premedication with glycopyrolate (0.1mg/kg s.c.) and Hypnorm ^{R} (fentanylcitrate 0,3mg/ml mixed with fluanizonum 10mg/ml, 0,3ml/kg i.m., Janssen Pharmaceutica, Beerse Belgium) the animals were anaesthetized with diazepam (2mg/kg i.v.) The laryngeal structures and the mobility of the cricoarytenoid joints were found normal at examination by means of a modified 4.0 mm paediatric laryngoscope (model 8576E, Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7 mm rigid endoscope (model 7218A). A digital video recorded on a computer was made of the VFs before and after the scarification

procedure (Richard Wolf video camera No 5512 and a Canopus ADVC100 digital video converter, Reading UK). The scarring procedure was performed with a 1.5 mm micro cup forceps (MicroFrance Medtronic, Düsseldorf Germany) excising the mucosa (Lp) and the superficial layer of the thyroarytenoid muscle.

Mesenchymal stem cell preparation and characterization

HMSCs were isolated and expanded from bone marrow (BM) taken from the iliac crest of healthy volunteers as previously described.[17,18] Heparinized BM was mixed with a double volume of phosphate-buffered saline (PBS), centrifuged at *900g,* resuspended and layered over a Percoll gradient (1.073 g/ml, Sigma-Aldrich, St Louis, MO, USA).The mononuclear cells were collected from the interface, washed and resuspended in hMSC medium consisting of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Life Technologies, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Life Technologies). The serum lot was selected on the basis of optimal cell growth and differentiation. The cells were plated in culture flasks (Becton Dickinson Biosciences, Bedford, MS, USA) at a density of 160.000 cells/cm^2. Non-adherent cells were removed after 48-72 hours and the adherent cells were cultured. When >70% confluence was reached, the cells were detached by trypsin and ethylenediaminetetraacetic (EDTA) (GibcoBRL, Grand Island, NY, USA) and replated at a density of 4000 cells/ cm^2. The cells were harvested in passage 5 and classified as mesenchymal stem cells based on their ability to differentiate into fat, bone and cartilage and by flow cytometric analyses (Positive to CD73, CD90, CD105 and CD166. Negative to CD14, CD31, CD34, CD45 and CD80).

Vocal fold hMSC injections

The hMSCs were centrifuged and washed from the growth medium in NaCl. Injections were made under video monitoring directly after the scarification procedure into the lamina propria and/or to the superficial part of the thyroarytenoid muscle of the VFs using a Medtronic Xomed laryngeal injector with a 27 gauge needle as previously described.[16,17,18] Eighteen scarred VFs were injected with hMSC in 0.1ml saline each. Each VF was injected with about 80.000-100.000 hMSC. Two scarred VFs were injected with only 0.1ml saline.

Four of the animals that received injection with hMSCs and the animal injected with only 0.1ml saline, were treated subcutaneously with immunosuppressant (Tacrolimus, (TC), 0.05mg/kg bw. s.c.) every second day. The dose was identical with our previous experiments.[16,17,18,24]

Sample procurement

After 4 weeks the animals were sacrificed with an overdose of sodium pentobarbital. Each larynx was dissected out and divided in the posterior midline. The larynges were placed in 4 % formaldehyde for histologic analyzes. Two VFs in the hMSC with TC group were damaged during the preparation and were left out. Four VFs from a previous pilot study, one identically treated with the hMSC group and one with the hMSC + TC group and two with the TC group were added for the histologic analyzes, grade of fibrosis and Lp-thickness. For the relative collagen type I measurements the samples from the pilot study were not included because different collagen probes were used in the studies as the original probe had left the market. Additional data for 5 normal VFs were collected from the data bank from earlier experiments, but were not used in the collagen measurements. Thus n for analyses of the Lp-thickness and

grade of fibrosis were, hMSC (without TC) n=11, hMSC + TC n=9, TC alone n=4, normal n=5, and for the collagen measurements hMSC (without TC) n=10, hMSC+ TC n=8, Tc alone n=2.

Histologic measurements

After fixation in 4% formaldehyde and 70% ethanol the VFs removed from the larynges were dehydrated and finally embedded in paraffin wax. Each VF was cut into 5µm thick horizontal sections covering the whole thickness of the VF. In average 30 sections per VF were generated. 2-4 slides from different levels of each VF were analyzed. Staining was made with hematoxylin-eosin for histological analyzes. Image analyzes were made at 10x or 20x magnification after digitization of the microscopic images. The slides were blindly analyzed at the Department of Pathology, Karolinska University Hospital Huddinge, Sweden. Intrareliability was assessed by blind reexamination of ten percent of the slides, randomly chosen. The results were identical.

Lamina propria thickness

Measurements of the lamina propria (Lp) thickness were made on the digitized hematoxylin-eosin images of each sample (custom made software Hans Larsson, Karolinska Institute, Dept. of Logopedics). The thickness of the Lp was measured at six spots covering the whole length of the VF. If a tendency to polyp formation was seen, the thickest part of the polyp area was included in the measurements. All measurement values were then used in the statistic evaluation.

Immunohistochemistry for Collagen type I staining

Staining was performed as previously described.[17,18] Briefly, slides were deparaffinised in xylene, rehydrated in alcohol and blocked in PBS containing 3% BSA. Slides were incubated with a primary antibody (No 90395, Abcam, Cambridge, UK), followed by incubation with a secondary antibody (nr.A11029 Alexa 488 anti-mouse, Invitrogen labs, Carlsbad, Ca. U.S.) Sections were rehydrated in ethanol and xylene and mounted with Vectashield containing DAPI (Vector labs Inc., Burlingame, CA). The relative contents of collagen type I in the VFs were measured from the digitized stains after a colour filtering and normalisation process with Photoshop (version 8.0) and a custom made software (Hans Larsson, Karolinska Institute, Dept. of Logopedics).

Fluorescence in situ hybridisation - FISH-analysis - for persistence of transplanted hMSC

Detection of human cells in the VFs was done with a human DNA specific reference probe, similar to an antibody, linked to a fluorescent molecule i.e. FISH-analysis.
The FISH-analysis was performed as previously described.[16] Briefly, slides were deparaffinised in xylene and rehydrated in alcohol, followed by pre-treatment with pepsin and hybridisation over night at 38°C with the human specific fluorescent probe (Spectrum Red human genomic DNA, Vysis Inc., Burlingame, CA). Six thousand cells were counted in randomly chosen sections from the optimal slides of each VF treated with hMSC. Detected human cells were summarized and related to the six thousand cells and the percental engraftment was calculated.

Statistical analyzes

For the Lp-thickness and Collagen I content findings, Kruskal-Wallis test was applied to test statistical differences. Differences between two groups were then assessed using Mann–

Whitney U test. For the Lp-thickness measurements each single value was included when differences between the various groups were estimated. For the relative Collagen I content the mean value between two series of measurements were used for each sample when differences between groups were calculated. For the histologic evaluation assessing grade of fibroses Fisher Exact test was used. Statistical significance was considered when $p < .05$

RESULTS

Hematoxylin-eosin staining, grade of fibrosis

The VFs were categorized into four groups depending on the grade of scarring, i.e. formation of fibrosis, using the following criteria[18]: Grade A - no or minimal signs of fibrosis, Grade B - a focal or non-compact fibrosis in the Lp or superficial vocal muscle, Grade C - a more compact fibrosis in the Lp and superficial muscle, Grade D - a compact fibrosis in Lp and superficial muscle as well as fibrosis in the deeper part of the vocal muscle. VFs treated with hMSC alone (n=11) were all except one compatible with group A or B (one was aligned to group C). The VFs given hMSC with immunosuppression (n=9) were placed in the B and C groups. The VFs treated with only TC (=4), were compatible with the C and D groups. Combination of groups A/B and C/D, respectively, yielded a significant difference between hMSC treated versus TC treated VFs ($p<.033$). No significant difference was shown between hMSC and hMSC+TC ($p<.26$), nor between hMSC+TC and TC alone, ($p<.081$). Furthermore, no significance was obtained between hMSC treated VFs and normal VFs ($p<.67$). In contrast, TC treated VFs showed significantly more fibrosis than normal VFs ($p=.048$). (Figure 1)

Lamina propria thickness

A significant reduction in lamina propria (Lp) thickness was seen for the hMSC treated VFs when compared with the VFs treated with TC alone ($p < .0001$).

There was a significant reduction for the hMSC treated versus the hMSC + Tc treated VFs ($p < .014$). A significant reduction was also shown for the VFs treated with hMSC+ TC compared with those treated with only TC, ($p < .002$). Between hMSC treated VFs and normal VFs a significant difference in the Lp thickness was found ($p = .008$). (Figure 2)

Collagen type I staining

Significant reduction in the relative content of collagen type I was found in the hMSC treated VFs without immunosuppressant (TC) compared with the VFs treated with only TC ($p = .026$). A significant difference was also found between hMSC treated VFs with and without TC ($p = .015$). No significant difference was seen between the VFs treated with hMSC + TC and the VFs treated with TC alone. One VF in the hMSC group and 2 in the hMSC+ TC group failed to mark in properly and were left out. Thus the compared groups were for the VFs treated with hMSC (without TC) n=9, hMSC+TC n=6 and TC alone n=2 (Figure 3)

FISH-analysis for Survival of hMSCs

The total survival rate of the injected stem cells, engraftment, was for both the VFs treated with or without immunosuppressant, 0.09%. For three of the VFs in the hMSC+TC group we found no positive cells in the FISH analysis. Among the VFs treated with hMSC without immunosuppressant we detected in half of the VFs 6 or fewer cells. Highest engraftment 0,58 % (or 35 cells) was fond in a VF in the hMSC+TC group. One VF in the group without TC showed a Fish stained cell in telofas indicating mitotic activity.

DISCUSSION

The aims of this study were to investigate if the Tacrolimus immunosuppression (TC) in the xenograft model, affects the survival of the hMSCs and also whether the immunosuppression per sé affects healing mediated by hMSCs injected into the injured VFs.

After one month of TC treatment, no significant impact upon the survival of the injected hMSCs was detected in this study. The engraftment after one month was in the present study half of what was found in one of our previous studies [24]. This finding may be due to donor related issues, such as the viability of the stem cells. Our finding of cells in division found in the non-immunosuppressed group, shows that the hMSCs have the potential to be active in the VFs for at least a month. This could indicate that immunosuppressant is not needed for hMSC studies in this model. It supports the assumption that the VFs represent a relatively closed compartment and might therefore be an immune-privileged site that for a limited time allows engraftment of xenogeneic cells.[25]

Histologic measurements comparing grade of fibrosis revealed significant differences between the VFs treated with hMSCs and those treated with only TC, the former showing significantly less fibrosis than the latter. There was no significant difference in grade of fibrosis between the VFs treated with hMSC with or without TC. However, when single samples were examined the fibrosis was observed to be more compact and widespread in the VFs treated with only TC when compared with the hMSC treated VFs both with and without TC. This indicates that hMSCs reduce the expansion of the fibrosis but their effect is restrained by the immunosuppression. (Figure 1)

The classification of fibrosis corresponded well with the measurements of Lp-thickness. The reduction in the Lp-thickness between the VFs treated with hMSC without TC and the VFs treated with TC alone was significant. Moreover the hMSC treated VFs with TC showed a

thicker Lp than those without TC. That the fibrosis in the Lp is less reduced with TC than without indicates that TC has a direct suppressive effect on the injected stem cells.

The measurements of the density of collagen type I were consistent with the classification of grade of fibrosis and the Lp-thickness measurements. The reduction in collagen type I for the hMSC treated VFs compared with the VFs treated with only TC is significant and so is the difference between the hMSC treated VFs with and without TC. Noticeable though is the non-significant difference between the hMSC treated VFs with TC and the VFs treated with TC alone. This also indicates that the anti-inflammatory effect of the hMSCs is repressed by the immunosuppression. We find that the results of this study support the assumption that the immunosuppressant used, Tacrolimus, did not contribute to the reduction of the fibrosis in the injured rabbit vocal folds, but suppressed the effect of the hMSCs.

CONCLUSION

In the xenograft rabbit model the immunosuppressant tacrolimus (TC) significantly reduced the anti-scaring effect of the injected hMSCs. However, following one month of immunosuppressive treatment with TC, no significant impact on the survival rate of the injected hMSCs was observed.

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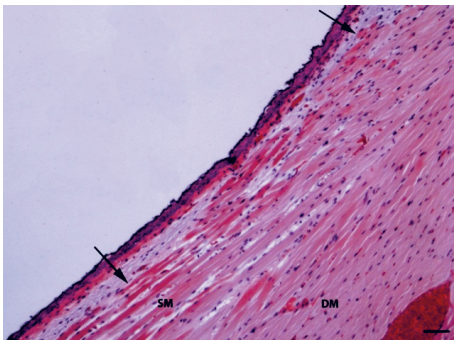
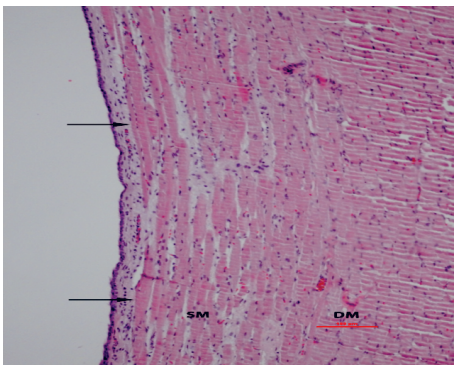
FIGURE LEGENDS

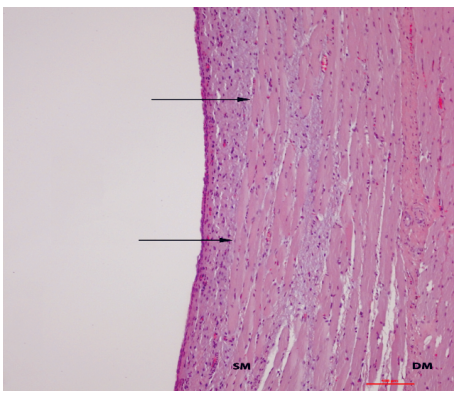
Figure1. A shows the hematoxylin-eosin staining of a normal vocal fold (VF), B hMSC treated VF, with only a tendency to loose connective tissue in the lamina propria (Lp), C of a hMSC-TC treated VF with loose connective tissue/ fibrosis, in the Lp and in the superficial muscle(SM), and D, a tacrolismus (TC) treated VF with compact fibrosis deep down in to the deep muscle (DM) and E, also a TC treated VF with less compact fibrosis in the Lp but extending into the SM, splitting up the muscle bundles of the thyroarytenoid muscle. Arrows mark the border between Lp and SM.



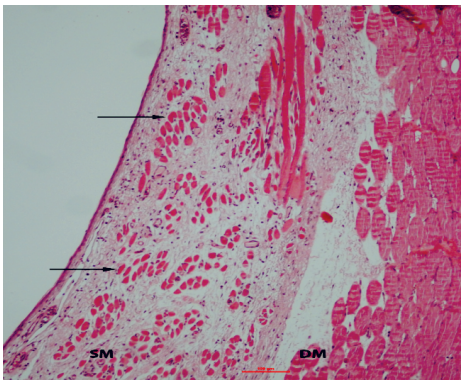
A, normal vocal fold, 10x magnification



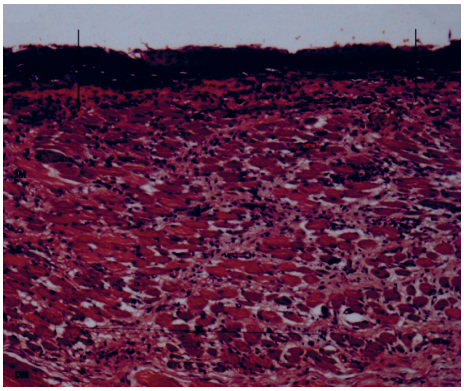
B, hMSC treated VF, 10x magnification



C, hMSC - TC treated VF, 10x magnification



D, TC treated VF, 10x magnification



E, TC treated VF, 20x magnification

Figure 2. Lamina propria (Lp) thickness (µm), in scarred vocal folds (VF) treated with human mesenchymal stem cells (hMSC) compared with folds treated with hMSC + immunosuppressant tacrolimus , (TC) and VFs treated with TC alone and normal VFs. All groups showed significant interdifferences when compared (* $p<.05$). Most reduction in Lp-thickness was shown in the VFs treated with hMSC alone and least reduction in the TC treated VFs. Each VF is measured at six spots including polyp formations if seen. Total measure points within brackets. Within boxes median, 25^{th} and 75^{th} percentiles; outside boxes 10^{th} and 90^{th} percentiles.

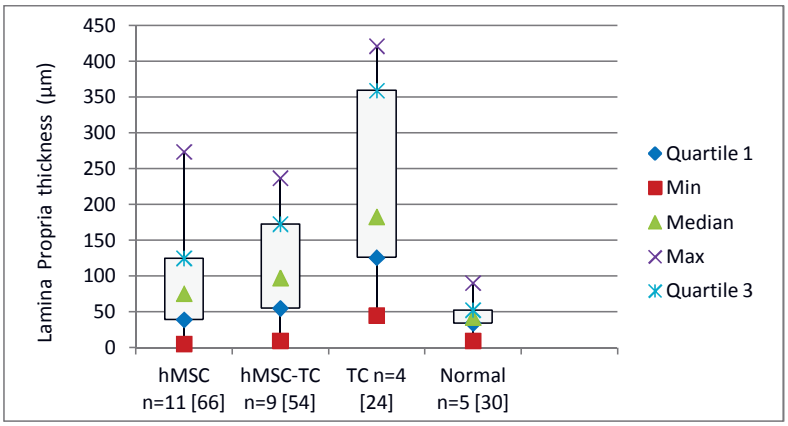


Figure 3. Relative collagen type 1 (%), in vocal folds (VF) treated with human mesenchymal stem cells (hMSC) compared with VF treated with hMSC + immunosuppressant, tacrolimus (TC) and VFs treated with onlyTC . The hMSC treated VFs showed significantly reduced amount compared with the VFs treated with hMSC-TC and those treated with only TC. There was no significant difference between the hMSC-TC and TC treated VFs. Within boxes median, 25th and 75th percentiles, outside boxes 10th and 90th percentiles. (* p<.05).