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Regenerative medicine of the airway cartilage: a morphological and immunohistochemical study with focus on cricoid cartilage defects treated with BMP 2

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ABSTRACT

Background. Cartilage provides flexible support for upper airway. Induction of cartilage healing is required for the success of many surgical interventions in the head and neck that are used as treatments for several diseases, such as subglottic stenosis and tumors of the larynx and thyroid.
The BMPs constitute a large family of factors that is part of the transforming growth factor beta superfamily. BMP-2 is a well-known modulator of skeletal patterning and, more specifically, of cartilage differentiation.
Aims. We have investigated the effects of rhBMP-2 on the regeneration of cricoid cartilage and of respiratory epithelium in a rabbit model. We have examined the integration between host cartilage and repair tissue induced by rhBMP-2. Furthermore, we have evaluated the role of cricoid perichondrium in the repair of cricoid cartilage defects treated with rhBMP-2. We have also attempted to determine what tissue or anatomical structure is a potential source of progenitor cells for the formation of new bone and cartilage induced by rhBMP-2. We have also evaluated the possible differences between young and adult rabbits in chondrogenesis and osteogenesis. We have evaluated the ultrastructural characteristics of new bone and cartilage in order to obtain a more detailed analysis of the cellular repair pattern.

Methods. The quantitative and qualitative characteristics of the healing pattern of the laryngeal wound were evaluated by histological techniques including histomorphometry, and immunohistochemistry. Tissue sections were stained with hematoxylin-eosin, alcian blue or periodic acid-Schiff for general morphological assessment (including the presence of chondrogenesis, osteogenesis, and general aspects of the vascular pattern); toluidine blue or safranine O/fast green for determining cartilage proteoglycan content; and they were immunostained with an antibody for tissue inhibitor of metalloproteinases 1 (TIMP 1), Ki 67 antibody, BMP receptor IB antibody, and BMP receptor II antibody. The distributions of collagen type I, collagen type II, and collagen type X were also determined. The ultrastructural characteristics of repair tissue were examined by means of TEM.

Results. Regeneration of both the epithelial layer and of cartilage was significantly better in rabbits treated with rhBMP-2. The cricoid cartilage defect was completely repaired by new bone and cartilage in rabbits treated with rhBMP-2 four weeks after surgery. Furthermore, there were no discontinuities or gaps at the margins of the cartilage defects. Proteoglycans were synthesized in newly formed cartilage, and they were present four weeks after surgery. Cartilage formation was only induced from host perichondrium that adhered to cricoid cartilage in rabbits treated with rhBMP-2. A cell proliferation marker (Ki-67) was strongly expressed in granulation tissue of specimens treated with rhBMP-2 and moderately expressed in muscle that was adjacent to cricoid cartilage in both control and rhBMP-2 treated specimens. BMP receptors were strongly expressed in cartilage, and moderately expressed in adjacent muscles. The areas of newly formed cartilage were not significantly different in young rabbits from those of adult rabbits, both at one week and at four weeks after surgery. However, the segments of cricoid perichondrium that responded to the rhBMP-2 were significantly larger in young rabbits than in adult rabbits. Collagen type I was strongly expressed in the fibrous layer of the host cricoid perichondrium of the adult rabbits, and it was moderately expressed in the fibrous layer of the young rabbits. Moreover, cricoid perichondrium had a larger proliferative zone in young rabbits. Ki-67 was expressed in the proliferative layer of cricoid perichondrium of young rabbits but not in adult rabbits.
TEM revealed that mineralized collage type I matrix, osteoblasts, and osteocytes were already present one week after surgery. Mineralized cartilage matrix invaded by newly formed blood vessels and osteoblasts were present two weeks after surgery. Well-structured bone trabeculae, and growth plate-like structures were present four weeks after surgery. New bone and cartilage were not induced in control rabbits.

Conclusions. rhBMP-2, delivered on an absorbable collagen sponge, induces the regeneration and the repair of rabbit cricoid cartilage defects. The relining of airway epithelium is more rapid when rhBMP-2 is administered than it is in control rabbits. The repair tissue induced by rhBMP-2 consists of new bone and cartilage integrated well with the host tissue. The newly formed cartilage matures and produces proteoglycans. Perichondrium of cricoid cartilage is a potent source of progenitor cells. Newly formed cartilage appears to originate only from the progenitor cells of the host perichondrium that adheres to cricoid cartilage in rabbits treated with rhBMP-2. New bone may originate from local skeletal muscle, and new chondrocytes support bone formation at the site of the cricoid cartilage defects.

rhBMP-2 induces temporal and quantitative patterns of new bone formation at the site of cricoid cartilage defects in young rabbits that are similar to those in adult rabbits, while chondrogenesis is qualitatively different. Appositional cartilage growth is more easily induced from the cricoid perichondrium of young rabbits than from the cricoid perichondrium of adult rabbits. Intramembranous and endochondral osteogenesis take place at the site of cricoid cartilage defects treated with rhBMP-2. Endochondral osteogenesis follows intramembranous osteogenesis. Progenitor cells of host perichondrium of cricoid cartilage form a growth plate-like structure similar to the epiphyseal growth plate.

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CONTENT

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their roman numeral.

I. **Ion Tcacencu**, Bengt Carlsöö, Pontus Stierna. Recombinant human bone morphogenetic protein-2 (rhBMP-2) enhances experimental laryngotracheal reconstruction in rabbits. Acta Otolaryngologica. 2004 Jun; 124(5):612-615.

II. **Ion Tcacencu**, Bengt Carlsöö, Pontus Stierna. Structural characteristics of repair tissue of cricoid cartilage defects treated with recombinant human bone morphogenetic protein-2. Wound Repair and Regeneration. 2004 May-Jun; 12(3):346-350.

III. **Ion Tcacencu**, Bengt Carlsöö, Pontus Stierna. Cell origin in experimental repair of cricoid cartilage defects treated with recombinant human bone morphogenetic protein-2. Wound Repair and Regeneration. In press, 2005.

IV. **Ion Tcacencu**, Bengt Carlsöö, Pontus Stierna. Effect of recombinant human BMP-2 on the repair of cricoid cartilage defects in young and adult rabbits: a comparative study. International Journal of Pediatric Otorhinolaryngology. In press, 2005.

V. **Ion Tcacencu**, Bengt Carlsöö, Pontus Stierna**,** Kjell Hultenby. Local treatment of airway cartilage defects with rhBMP-2 induces growth plate-like morphology of chondrogenesis. Submitted to Bone, 2005.

LIST OF ABBREVIATION

TGF-beta	transforming growth factor beta
BMPs	bone morphologenetic proteins
rhBMP-2	recombinant human bone morphogenetic protein-2
HE	hemotoxylin-eosin
AB-PAS	alcian blue-periodic acid-Schiff
CS	absorbable collagen sponge
PGs	proteoglycans
TIMP	tissue inhibitor of metalloproteinase
MPC	mesenchymal progenitor cell
BMPR	bone morphogenetic protein receptor
DAB	diaminobenzidine
MMP	matrix metalloproteinase
ALP	alkaline phosphatase
TEM	transmission electron microscopy
OPN	osteopontin

Introduction

Cartilage wound healing

Cartilage structure

Cartilage is a specialized connective tissue. It is found in various parts of the human body - in joints, ribs, the larynx, trachea and bronchi, nose, and ears.

Most of the skeleton is cartilaginous at early stages of fetal development. This cartilage is known as temporary cartilage and is subsequently replaced by bone. This contrasts with permanent cartilage, which remains unossified throughout life.

Cartilage is composed of chondrocytes surrounded by extracellular matrix. Cartilage is particularly rich in extracellular matrix: it constitutes 90% of the dry weight of the tissue[1], while chondrocytes occupy only 1%-10% of the volume.

The chondrocyte is spheroid, with a diameter of about 20 µm. It has a lower metabolic rate than most other cell types, largely because its location is characterised by a low oxygen concentration: the pO_2 is less than 5%.

Chondrocytes have cell appendages that extend a short distance into the matrix, but do not touch other cells. Cell-matrix interactions are therefore essential in cartilage for the maintenance of the extracellular matrix. Light microscopy shows that chondrocytes reside within compartments called lacunae.

Several types of cartilage have been defined: hyaline cartilage (nasal septum, tracheal rings, cricoid cartilage, thyroid cartilage, articular cartilage), elastic cartilage (auricule, epiglottis), and fibrocartilage (meniscus). The classification is based on the presence of collagen and elastic fibers, and on tissue morphology[2].

The hyaline cartilage matrix contains hyaluronic acid, proteoglycans (PGs) and collagen fibers that are mainly of type II[3] (Figure 1). These components give unique mechanical properties to hyaline cartilage.

Hyaline cartilage from different anatomic sites has different compositions of PGs and collagen. For example, hyaline cartilage in the joint and hyaline cartilage in the trachea and cricoid differ in their ratios of collagen type I to collagen type II[4, 5].

Type I collagen is a major component of fibrocartilage, skin, bone, dentin and tendon. Collagen type I is composed of three polypeptide chains, as is collagen type II[6].

The network of collagen fibers provides both tensile strength to the tissue and the ability to contain the swelling pressure of the embedded PGs.

PGs are a group of glycoproteins that contain a core protein to which one or more glycosaminoglycans is or are attached. Cartilage tissue is very rich in PGs. These molecules play structural and regulatory roles, and can act as an extracellular reservoir for growth factors and for hormones[7]. Deposition of PGs is considered to be a marker of chondrogenesis.

The predominant proteoglycan present in cartilage is aggrecan - a large chondroitin sulfate proteoglycan[8]. Aggrecan consists of a large protein core to which many chondroitin sulfate and keratan sulfate side-chains are attached. The side-chains create a hydrophilic environment, and this ensures that the entire aggrecan structure is well hydrated and that it has load-bearing and compressive properties.

Aggrecan is present not only in cartilage but also in the aorta, intervertebral disc and tendons.
The perichondrium is a membrane of dense connective tissue that surrounds airway cartilage. It contains blood vessels but no nerves.

Duynstee et al.[9] pointed out that the perichondrium has two different layers. They suggested that cells of the inner (cambium) layer, located closest to the cartilage, are the source of progenitor cells, which are capable of inducing new cartilage. They showed that the outer layer consists predominantly of more fibrous tissue.

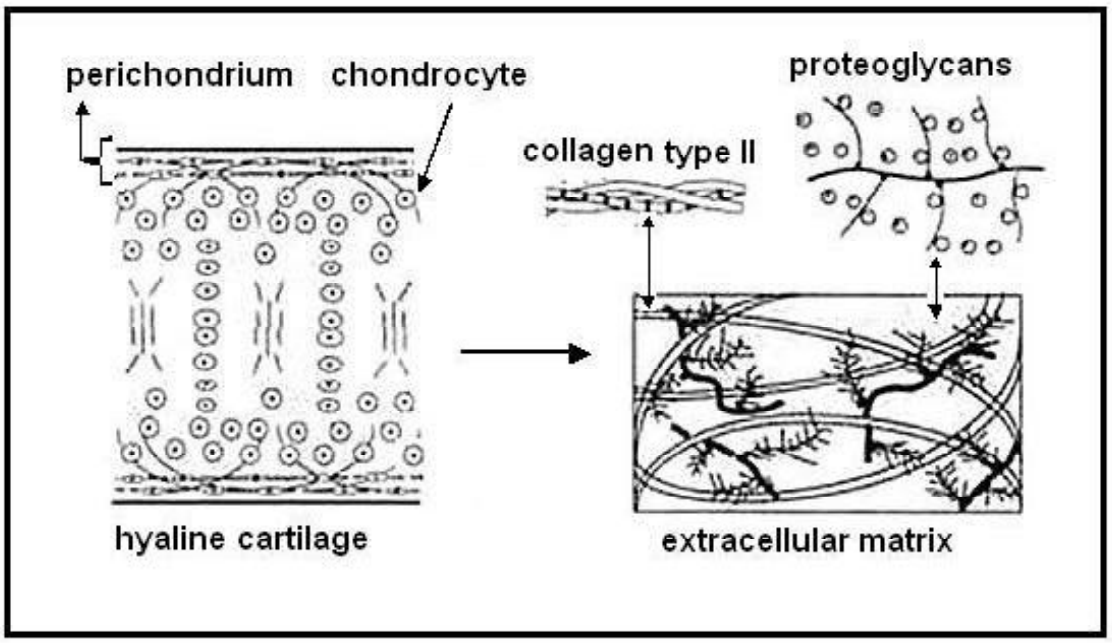

Figure 1. Hyaline cartilage structure.

Cartilage repair and regeneration

Cartilage offers unique challenges in terms of repair. The cartilage is hypocellular and the tissue must therefore rely on autocrine and/or paracrine communication between cells. Cartilage contains no blood vessels and relies on diffusion for its nutrient supply. Furthermore, cartilage lacks the progenitor cells and growth factors needed for tissue regeneration, probably because it has no vascular supply. Unlike bone, the wound healing potential of cartilage is poor or even absent in adults[1].

Small defects in cartilage can self-repair, but large defects cannot. Injury to hyaline cartilage usually leads to the deposition of fibrous scar tissue or fibrocartilage. Scar tissue is usually deposited faster than the cartilage can repair itself.

The regeneration of cartilage involves the proliferation and differentiation of progenitor cells into chondroblasts and chondrocytes, which elaborate proteoglycans and collagen type II fibrils.

Induction of cartilage healing is important for the success of many surgical interventions in the head and neck. Such interventions include laryngotracheal reconstruction, which is used as a surgical treatment for several laryngotracheal diseases, such as subglottic stenosis and malignant diseases of the larynx and thyroid.

The surgical repair of subglottic stenosis remains one of the most challenging aspects of airway management. The airway can be repaired using cartilage grafts in cricoid split procedures. Autologous cartilage grafts are most frequently used to increase the airway diameter and to provide a rigid framework. Costal cartilage is most commonly used for mature stenoses. Auricular cartilage has also been used in laryngotracheal reconstruction[2].

Unsatisfactory integration of repair tissue and the loss of grafting material through absorption are two problems in cartilage regeneration after trauma or surgery.

Age-related changes in cartilage wound healing

Several studies on wound healing of cricoid cartilage have shown that there is a relationship between the pattern of wound healing and age[3, 4]. Cherukupally et al.[5] showed that there was a progressive, age-related attenuation of staining for markers of chondrocyte proliferation in the cricoids of 8-week-old and 1.5-year-old rabbits after a cricoid injury. This attenuation did not take place in 4-week-old rabbits.

Wagner et al.[6] demonstrated that neonatal rat cartilage has the capacity for rapid scar-free regeneration after full-thickness incision, while adult cartilage does not.

Regenerative medicine – a new healing paradigm

The cost of tissue damage due to regenerative incompetence is very high. Thus medical science seeks ways not only to prevent and cure disease, but also to restore the structure and function of damaged tissues and organs.

Much research is currently being carried out into the possibility of replacing damaged body parts. This research constitutes a new field of medicine known as regenerative medicine.

It is necessary to understand the biology of regeneration in order to establish regenerative medicine[7]. The objective of regenerative biology is to define the factors that lead to a regenerative response and how these factors differ during a fibrotic response to injury. Regenerative medicine then seeks to apply this knowledge to develop therapies that will stimulate the functional regeneration of damaged human tissues that do not regenerate spontaneously, or whose regenerative capacity has been compromised.

Strategies

Potential strategies of regenerative medicine include stem (progenitor) cell transplantation, implantation of bioartificial tissues synthesized in the laboratory (tissue engineering), and the induction of regeneration from the body's own cells by enhancing the injury (wound) environment (by adding growth factors, for example) and/or the regeneration competence of responding cells.

There are two categories of stem cells: pluripotent embryonic stem cells derived from blastocysts, and restricted lineage (unipotent to multipotent) adult stem cells. Stem cells in both categories are distinguished from one another and from their progeny by a combination of location, morphology, cytology, cell surface receptors, and transcription factors.[8]

Advances in cell biology, developmental biology and molecular biology, and the discovery of progenitor (regeneration-competent) cells in many non-regenerating mammalian tissues, have stimulated systematic investigations that will enable us to regenerate these tissues by cell transplantation or by the pharmaceutical induction of regeneration from the body's own tissues. Many research studies aim at the identification of the soluble and insoluble signals, and their transduction pathways, that regulate the proliferation and differentiation of progenitor cells, and the signals that inhibit their activity after injury.

The role of growth factors

Growth factors are signal proteins that act in a paracrine or autocrine way to control wound healing, tissue regeneration, and normal body development. Growth factors are released from local tissue or blood and are directly involved in cell growth, cell differentiation, inflammation, and tissue repair. The action of growth factors depends strongly on the tissue-specific microenvironment. Growth factors may accelerate the wound healing of airways[9].

The direct application of growth factors on airway wounds has been examined in several studies. For example, airway epithelial regeneration is enhanced by basic fibroblast growth factor[10] and by transforming growth factor beta-3[11].

Zahm et al.[12] showed that hepatocyte growth factor can improve the wound repair process of the respiratory epithelium in vitro by increasing cell migration. It does not, however, increase cell proliferation. Katic et al.[13] studied the effect of osteogenic protein-1 on airway cartilage repair in a dog model of thyroid cartilage defects. Osteogenic protein 1 induced the formation of bone, cartilage and ligament-like tissue. This study did not, however, examine the effects of osteogenic protein-1 on airway epithelium regeneration.

Treatment with vascular endothelial growth factor in a rabbit laryngotracheal reconstruction model does not improve autologous cartilage graft survival[14].

Bone morphogenetic proteins

Classification and structure

Bone morphogenetic proteins (BMPs) are differentiation factors that induce activation, migration to the site of the BMP implant, and differentiation of MPCs into bone forming and cartilage-forming cells[15].

BMPs belong to the TGF-beta superfamily, which is a group of related peptide growth factors. More than 40 members of this family have been identified, including BMPs, growth and differentiation factors (GDFs), inhibins/activins, TGF-betas and Mullerian-inhibiting substance[16]. Members of the TGF-beta superfamily are synthesized as large precursor

molecules, and the mature protein is released from a propeptide region by proteolytic cleavage.

BMPs are dimers, the two monomers of which are joined by several disulphide bonds. BMPs are active both as homodimer molecules, which consist of two identical chains, and as heterodimer molecules, which consist of two different chains.

Fifteen BMPs have been identified, and they have been classified into subfamilies based on their amino acid sequence similarities. The first subgroup contains BMP-2 and BMP-4, highly related molecules that differ mainly in the N-terminal region, with BMP-2 containing a heparin-binding domain. BMP-5, BMP-6, BMP-7, also known as osteogenic protein-1 (OP-1), and BMP-8 (OP-2) are members of the second subgroup. These are slightly larger proteins than BMP-2 and BMP-4, and there is approximately 70% amino acid sequence identity between the subgroups. The third subgroup includes BMP-3 (osteogenin), and GDF-10, a related growth factor.

Bone and other tissues contain only low concentrations of BMPs and BMP precursor molecules. The biological activity, biochemistry, and three-dimensional structures of BMPs have been studied mainly in recombinant BMPs. Native BMPs are much more heterogeneous in size and glycosylation pattern than recombinant BMPs[17]. Most of the human BMPs have been expressed in mammalian cells using a recombinant DNA biotechnology process[18]. Each cell line produces a single BMP molecule, which is secreted into the medium in which the cells are growing. Some research groups have produced BMPs from bacterial expression systems[19]. Bacterial expression systems have higher productivities and protein yields than eukaryotic systems.

Activities of BMPs

The chondrogenic and osteogenic activities of BMPs are directed towards immature and potent cells. BMPs bind to a complex of transmembrane serine/threonine kinase receptors and initiate a cellular signaling cascade via this complex. The complex consists of type II receptors (BMPR-II) and type I receptors (BMPR-IA, BMPR-IB)[20, 21] (Figure 2). The type II receptors are involved in initial ligand binding, and type I receptors are subsequently recruited to form a heterotetrameric complex. The type I receptors are transphosphorylated by the kinase domain of the type II receptors in this ligand/receptor complex, resulting in the initiation of downstream signaling. The ligand interaction differs for BMP receptors because both type I and type II receptors are capable of low-affinity interaction with BMPs, but only ligand binding of both receptors results in a high-affinity heterometric ligand-receptor complex capable of BMP dependent signaling[22].

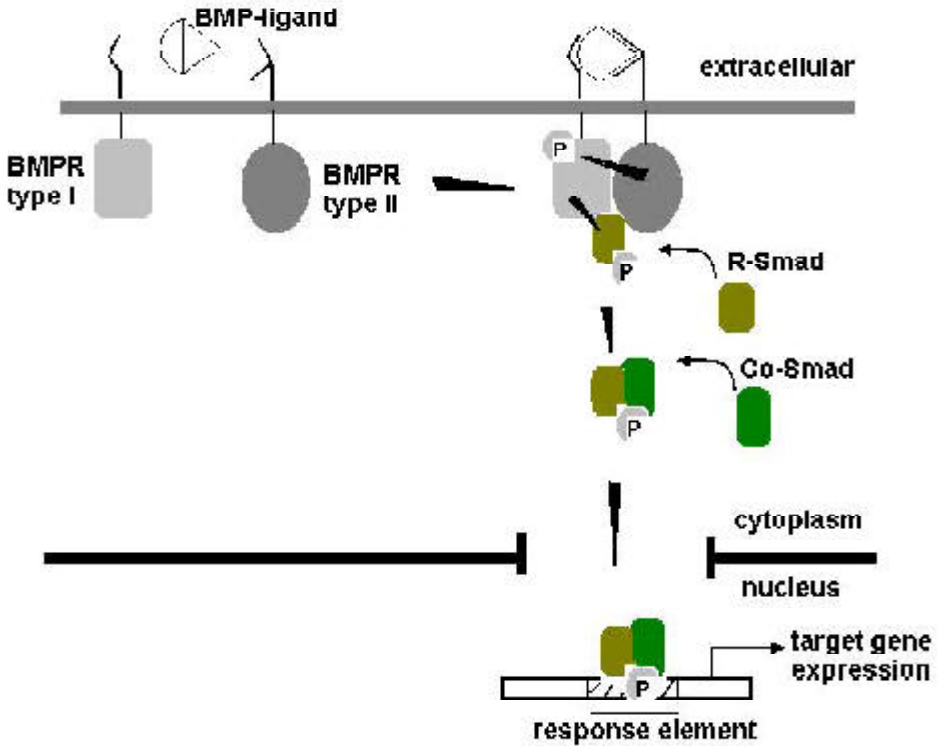

Figure 2. BMP signaling pathway (modified from Balemans et al.[35]).

The activated BMP type I receptor then phosphorylates a member of the Smad family of intracellular proteins. Smad proteins are the functional signal transducers of the TGF beta/BMP family[1]. They can be classified into three subtypes according to their structure and mechanism of action. The first subtype, receptor-regulated Smads (R-Smads), includes Smad1, Smad5, and Smad8, and these are directly phosphorylated and activated by a BMP type I receptor. The second subtype includes Smad2 and Smad3, which are phosphorylated and activated by activin or TGF-beta type I. Smad1 and Smad5 form heteromeric Smad Smad complexes with Smad 4 (Co-Smad). The Smad complexes translocate into the nucleus and participate in the regulation of target genes. This occurs predominantly by the binding of other transcription factors and transcriptional regulators.

The third subtype of Smad interferes with the activation of R-Smads. Members of this class are termed inhibitory Smads (Smads 6 and 7)[2].

Each BMP can be tested by implantation in vivo into a bone induction assay system. This assay has been used to show that BMP-2 induces the formation of new cartilage and bone tissues, demonstrating that the implantation of an individual BMP is sufficient to start the entire osteoinductive process.

Approximately 2 µg of BMP can be extracted from each kilogram of powdered normal bone[27]. In contrast, the concentrations of BMP required to accelerate fracture healing range from 0.01 mg/ml in rodents to 1.5 mg/ml in non-human primate models[3]. Supraphysiological concentrations of BMPs may be required to overcome the effect of inhibitors of BMPs.

The activity of BMP is controlled at many levels: intracellularly, at the membrane site, and extracellularly[4]. Several extracellular BMP antagonists have been isolated in vertebrates, including noggin and chordin. BMP activity is also regulated by inhibitory Smads in the cell cytoplasm. Further, pseudoreceptors at the membrane counteract the effects of BMPs[5]. Cartilage and bone induction can take place only at locations where BMP is present, and it can take place only during the time that BMP is present[6].

The role of BMP carriers

BMP can induce bone when added in solution[7], not bound to a carrier, but the dose needed to induce bone formation is much lower when BMP is administered on an appropriate carrier. The carrier acts as a scaffold for cell invasion and retains BMP at the site of delivery. Several types of carrier material are used for BMP delivery. They include inorganic materials, synthetic polymers, natural polymers, and composites of the first three materials [34].

Natural polymers include collagen (demineralized bone matrix, fibrilar collagen), hyaluronans, fibrin, alginate, and other animal-derived or plant-derived polysaccharides. The main disadvantages of natural polymers are their potential immunogenicity and the risk of disease transmission. The use of recombinant forms of natural polymers can eliminate these disadvantages.

Collagen has been the most commonly used natural polymer carrier. Collagen implants and dressings have also been used as hemostats, tissue and bone fillings, surface dressings for wounds, tooth repair, the repair of vascular, corneal and urological defects, and for controlled drug delivery[8]. Most implants are derived from bovine collagen, usually hide or tendon collagen. The collagen from these sources consists predominantly of type I fibrous collagen, with a small proportion of type III collagen.

The degradation of a collagen implant occurs as a normal process of remodeling. Degradation is caused by infiltrating cells, and the process is regulated by cell-matrix interactions in which enzymes are produced. Cellular infiltration occurs in a sequential manner, with the infiltration by neutrophils, monocytes and lymphocytes increasing during the first six days following injury, and the infiltration of fibroblasts occurring during the complete period. The fibroblasts eventually predominate. The collagen implant is removed by enzymatic degradation. Collagen is degraded by endopeptidases, such as metalloproteases, serine proteases, cysteine proteases, and aspartic proteases.

We chose absorbable collagen sponge as the carrier for rhBMP-2. This sponge is a hemostatic sponge made from bovine tendon-derived type I collagen, and it has been approved for surgical implantation. Ma et al.[9] compared the properties of collagen, hydroxyapatite, tricalcium phosphate, glass beads and polymethylmethacrylate that make them suitable as carriers, and concluded that collagen is the optimal delivery system for BMPs. Other studies have shown that collagen is an excellent delivery system for growth factors in the wound repair of soft tissues and hard tissues[10].

The absorption of rhBMP-2 implanted with a biomaterial carrier generally takes place in two phases: an early and rapid rhBMP-2 loss (within a few hours of implantation), followed by a more gradual rhBMP-2 loss[11]. Collagen sponges retain the highest fraction of the implanted dose of rhBMP-2 and lose rhBMP-2 gradually from the implant site[12]. A higher local retention of rhBMP-2 yields a higher osteoinductive activity[13].

The safety profile of BMPs

rhBMPs are currently being evaluated for clinical use, and appear to be effective in enhancing the bone reparative process. The clinical trials have been accompanied by a detailed determination of safety using both in vitro and in vivo assays[14]. The safety issues examined include the cancer risk, systemic toxicity, reproductive toxicity and immunogenicity.

The safety data available for rhBMP-2 show no systemic adverse effects, and they show a low risk for carcinogenesis. Formation of antibodies against rhBMP-2 is similar to that for control subjects. There is no evidence of maternal toxicity, embryolethality, fetotoxicity, or teratogenicity.

Perspective

Bone regeneration with BMPs in animals has been generally successful, but recent human studies have shown a large variation in individual responses[25].

Furthermore, the ectopic bone formation induced by BMPs is age-related. Nagai et al.[15] have shown that the rate and quantity of ectopic bone formation induced by purified bovine BMP are reduced in aged rats.

The successful administration of BMP to a patient depends on effective dosing[16]. It is difficult to control the quantity of the recombinant BMP that is necessary for a clinical effect at the site of the graft. The effect of BMP depends on the conditions at the site of the graft, such as the supply of local and systemic growth factors and hormones, and the presence of target cells.

Future studies will focus on the development of carrier materials that have mechanical properties that make them appropriate for the controlled release of BMPs. Such materials must, naturally, be practical in surgical procedures.

Tissue inhibitors of metalloproteinases

The matrix metalloproteinases (MMPs) form a family of enzymes that can degrade various components of the extracellular matrix. MMPs act primarily on the cell surface and in the extracellular space, and the activities of MMPs are controlled by endogenous inhibitors such as alpha2-macroglobulin and the tissue inhibitors of metalloproteinases (TIMPs)[17].

TIMPs are considered to be the key inhibitors of metalloproteinases in tissue. The balance between MMPs and TIMPs may play a critical role in the regulation of the turnover of the extracellular matrix. Disturbance of this balance has been implicated in a variety of pathological states, including osteoarthritis, tumor invasion, and inadequate cartilage repair.

TIMPs are multifunctional proteins, and the mechanisms underlying the cellular effects of TIMPs are not well understood.

Frenkel et al.[18] showed that the level of TIMP-1 mRNA increases in cultures of bovine articular chondrocytes treated with BMP-2. Immunohistochemical staining revealed that TIMP-1 is produced in rabbit cartilage defects treated with BMP-2. It appears that the effect of BMP-2 is mediated by regulating the level of TIMP-1 in tissue.

Aims

The aim of the work presented in this thesis was to investigate the effects of rhBMP 2 on the regeneration of airway cartilage and the regeneration of respiratory epithelium in a rabbit model of a cricoid defect.

We have examined the structural characteristics of repair tissue induced by rhBMP 2, including cartilage PG content, general aspects of the vascular pattern, the pattern of TIMP-1 expression, the integration between the host cartilage and new bone, and the integration between the host cartilage and newly formed cartilage tissue.

Moreover, we have determined the role of cricoid perichondrium in the repair of cricoid cartilage defects. We have also attempted to determine what tissue or anatomical structure may act as a source of progenitor cells for the formation of cartilage and

bone induced by rhBMP-2 at the site of cricoid cartilage defects.

We have also evaluated the possible differences of the chondrogenesis and the osteogenesis induced by rhBMP-2 at the site of cricoid cartilage defects in young and adult rabbits. Furthermore, we have investigated the ultrastructural characteristics of new bone and new cartilage induced at the site of cricoid cartilage defects. We wanted to obtain a more detailed analysis of the cellular repair pattern, such that we could determine whether both direct and indirect osteogenesis occur at the site of cricoid defects.

Materials and Methods

Animal models

The work presented in this thesis used a rabbit model of a cricoid defect (Figure 3). The structure of the larynx of rabbits is similar to that of man: in particular, the organization of the different cartilaginous parts and their interrelations are similar, as are the structure of the respiratory epithelium and the subepithelium with its associated vessels, glands, and the elastic system[19].

Our model corresponds broadly with the model described by Walner et al.[20]. Briefly, the larynx and the trachea were exposed through a vertical midline skin incision. The cricoid cartilage was split vertically in the anterior midline, and a vertical strip of width 2 mm was excised from the anterior part of the cricoid cartilage. A piece of commercially available bovine collagen sponge (Helistat, Integra Life Sciences Corp., Plainsboro, NJ) with dimensions 5 x 3 x 3.5 mm^3 was inserted into the cartilage defect. The collagen implant was soaked with glutamate-glycine buffer or with 20 ml of rhBMP-2 solution (0.25 mg/ml).

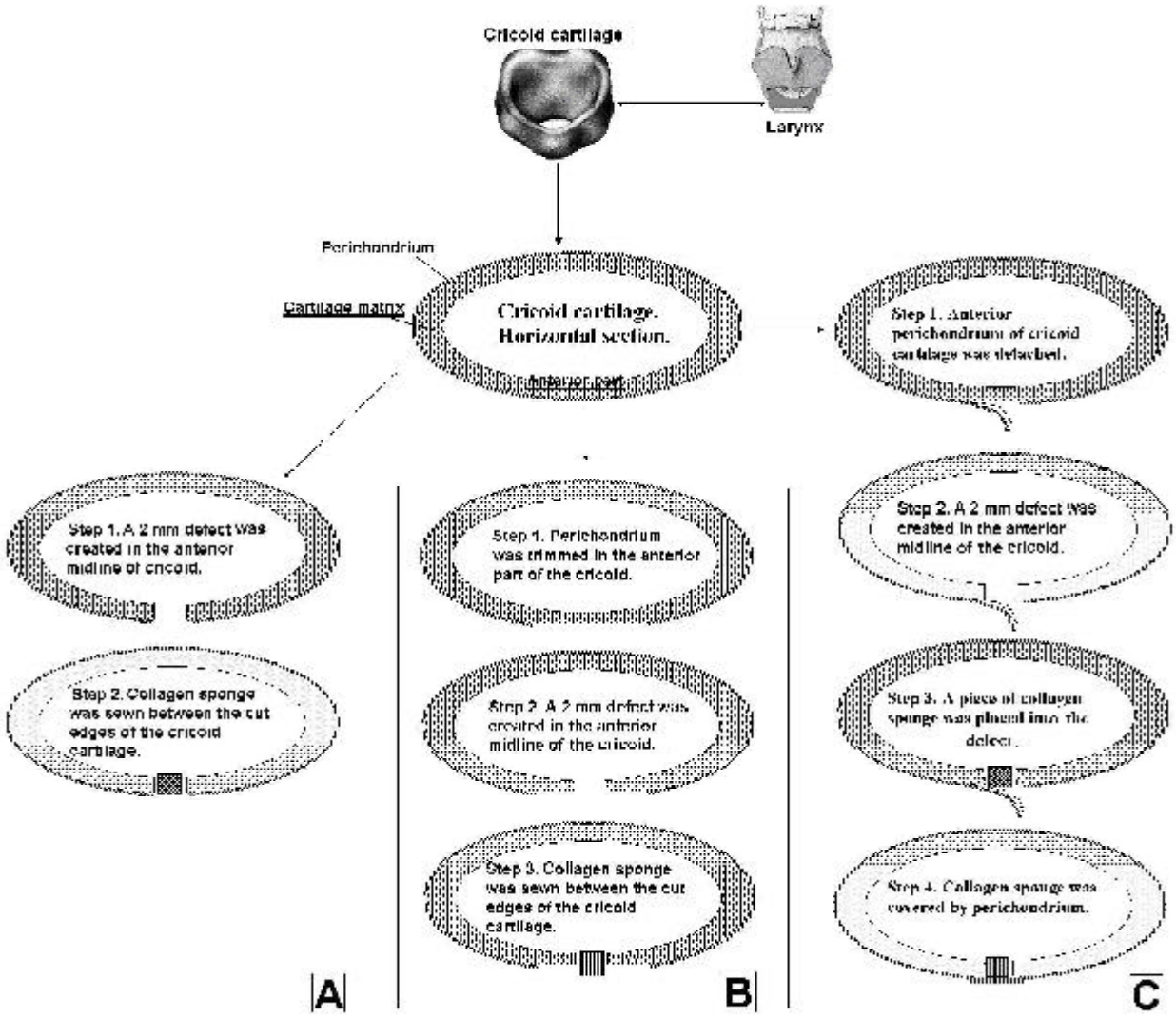

Figure 3. Diagram of the surgical techniques. A, see Papers I, II, IV and V; B, and C, see Paper III.

The dose, 5 µg rhBMP-2 per implant, was chosen based on published data on the use of rhBMP-2 for the treatment of defects of articular cartilage in rabbits[1, 2].

Each collagen sponge was sewn between the cut edges of the cricoid cartilage with a single Ethicon® suture, thereby stenting the defect. The cervical incision was closed layer by layer. The rabbits were killed one week, two weeks or four weeks after surgery.

The procedure was modified for the cell origin study (Paper III) in order to be able to evaluate the role of cricoid perichondrium in cartilage regeneration. Perichondrium from the anterior part of the cricoid cartilage was trimmed off or was detached as a myo-perichondrial flap and was subsequently used. Otherwise the model was the same as the model used in other studies

Tissue preparation

The larynges were surgically removed at the specified time and fixed in 4% formalin for 24 hours. The specimens were decalcified and embedded in paraffin, then serially sectioned with a microtome along a vertical axis at 100-200 µm intervals, starting at the inferior border of the cricoid cartilage. The sections, each of thickness 5 mm, were placed on glass slides and stained with HE and AB-PAS, for general morphological and vascular assessment; with toluidine blue for evaluation of neochondrification; or with safranine O/fast green for assessment of PG content. We used the intensity of the safranine O/fast green staining to measure the amount of PGs present in the cartilage, by visual assessment using a grading scale (Paper II).

The larynges intended for histochemistry and immunohistochemistry were surgically removed at the specified times, embedded in Tissue-Tek OCT compound (Sakura Finetek Europe BV, The Netherlands), and frozen in liquid nitrogen. Sections of thickness 7 µm were cut from each frozen block of tissue.

The distribution of calcified matrix and the localization of alkaline phosphatase (ALP) activity after rhBMP-2 treatment were studied by histochemistry (Papers III and IV).

We used the Alizarin red method to identify calcified matrix. Calcium forms an orange-red pigment with the dye Alizarin red S. Cryo-sections were incubated in a 2% aqueous solution of Alizarin red S for one minute, rinsed in acetone for 45 seconds, rinsed in xylene for one minute, and then mounted.

Fresh cryo-sections destined for the detection of ALP activity were incubated for 15 minutes in a solution of naphtol AS-BI mixed with fast red TR (Sigma) (1:1). After washing with distilled water, the sections were counterstained in Alcian blue for five minutes. Regions containing ALP activity appeared red. Sections were incubated with fast red solution without naphthol AS-BI as a negative control.

Histomorphometry

The healing patterns of the laryngeal wounds were evaluated by histomorphometry (Paper I). The histomorphometric measurements were carried out using an eyepiece micrometer in an Olympus BX40 microscope. We measured the sizes of the initial cartilage defect, the segment of mucosa without epithelium lining, the area of neochondrification and the area of new bone formation.

In the age-related study (Paper IV), five sections from each paraffin-embedded specimen and two sections from each frozen specimen were used for the evaluation of neochondrification by histomorphometry. We measured the area of newly formed cartilage and the segments of cricoid perichondrium that responded to rhBMP-2 by chondrogenesis, irrespective of the relative difference in cricoid lumen size and cricoid cartilage thickness in young and adult rabbits. The histomorphometric measurements were made on each section using IP Lab Spectrum image-analysis software (Signal Analytics Corporation, USA).

The data was statistically analyzed by the Mann-Whitney U-test. Significance was defined as $p < 0.05$.

Immunohistochemistry

Cryosections were fixed in acetone for ten minutes, then immunostained with mouse anti-human TIMP-1 monoclonal antibody (Chemicon International, Inc., 1:50 dilution), mouse anti-human Ki-67 antibody (DakoCytomation Denmark A/S, 1:100 dilution, proliferating cell nuclear antigen antibody), goat anti-human BMPR-II antibody (R&D Systems, Inc., 1:10 dilution), goat anti human BMPR-IB antibody (Santa Cruz Biotechnology, Inc., 1:100 dilution), mouse monoclonal anti-collagen type I antibody (Sigma-Aldrich Sweden AB, 1:1000 dilution), mouse monoclonal anti-collagen type X antibody (Sigma-Aldrich Sweden AB, 1:1000 dilution), and mouse monoclonal anti collagen type II antibody (Calbiochem, Inc., San Diego, concentration 1 µg/ml).

Sections were incubated with the primary antibody for one hour in a humidified chamber at room temperature. A rabbit anti-goat antibody conjugated with horseradish peroxidase and a goat anti-mouse antibody conjugated with peroxidase-labeled polymer (DakoCytomation Denmark A/S) were used as secondary antibodies. Peroxidase reactions were then visualized using a commercial DAB-peroxidase kit (DakoCytomation Denmark A/S). Finally, sections were counterstained with hematoxylin and mounted for light microscopy.

The posterior part of the cricoid cartilage ring and the adjacent muscles were used as normal tissue control (Paper III).

Immunohistochemical staining was graded on a scale on which "-" specifies no staining, 1+ specifies moderate to intense staining in less than 1/3 of the cells, 2+ specifies moderate to intense staining in 1/3 to 2/3 of the cells, and 3+ specifies moderate to intense staining in more than 2/3 of the cells. Immunostaining was independently scored for host cricoid cartilage, host cricoid perichondrium, granulation (fibrous) tissue, airway epithelium, muscle adjacent to cricoid cartilage, newly formed cartilage, and new bone (including bone marrow).

Transmission electron microscopy

The cricoid perichondrium and host cricoid cartilage, the cricoid defect region including the newly formed cartilage, and new bone tissue were selected and prepared for the TEM examination (Paper V).

The specimens were fixed in 2% glutaraldehyde + 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM $CaCl_2$, pH 7.4 at room temperature for 30 min, followed by 24 hours at 4 °C. Specimens were rinsed in 0.15 M sodium cacodylate buffer containing 3 mM $CaCl_2$, pH 7.4, and then post-fixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM $CaCl_2$, pH 7.4 at 4 °C for 2 hours. The specimens were subsequently dehydrated in ethanol followed by acetone, and then embedded in resin LX-112 (Ladd, Burlington, Vermont, USA).

Semi-thin sections were cut and stained with toluidine blue and used for light microscopic analysis. Ultra-thin sections (approximately 40-50 nm) were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10/Leo 906 transmission electron microscope at 80 kV.

Results

One week after surgery

Appositional cartilage growth was present one week after surgery in rabbits treated with rhBMP-2. Furthermore, the segment of the larynx mucosa that lacked epithelial lining was significantly smaller in specimens treated with rhBMP-2 than it was in control specimens. There was no difference in the infiltration of inflammatory cells at the site of the cricoid cartilage defect between rabbits treated with rhBMP-2 and those that were not treated (Paper I).

The repair tissue in rabbits treated with rhBMP-2 consisted of islands of newly formed cartilage located in the vicinity of host perichondrium, some granulation tissue, and the remainder of the collagen sponge (in the center of the defect area) (Paper II).

Chondrogenesis had not occurred at the sites of cricoid cartilage where the perichondrium had been removed. Newly formed cartilage was present one week and four weeks after surgery only at the sites where cricoid perichondrium was left intact in rabbits treated with rhBMP-2. Cricoid perichondrial flaps that covered the cartilage defects were not able to induce the growth of cartilage in rabbits treated with rhBMP 2 nor in control rabbits (Paper III).

Alizarin red S staining revealed irregularly shaped calcified matrix at the margin of the newly formed cartilage. ALP activity was present in the newly formed cartilage one week after surgery (Paper III). ALP was also present in the submucosa of both control specimens and rhBMP-2 treated specimens one week and four weeks after surgery.

The patterns of expression of the BMP receptors, BMPR-IB and BMPR-II, were similar one week and four weeks after surgery (Paper III). The patterns of expression of the BMPRs were similar in control specimens, in specimens treated with rhBMP-2, and in normal larynx tissue. BMPRs were strongly expressed in host cricoid cartilage, cricoid perichondrium, and newly formed cartilage. They were moderately expressed in adjacent muscles.

The cell proliferation marker Ki-67 was strongly expressed in the granulation tissue of specimens treated with rhBMP-2 one week after surgery (Paper III). Ki-67 was moderately expressed in muscles adjacent to the cricoid both in control specimens and in specimens treated with rhBMP-2. Ki-67 was also

moderately expressed in the airway epithelium of specimens treated with rhBMP-2, and it was expressed to a lesser extent in the airway epithelium of control specimens and of the normal larynx.

Ki-67 was not expressed in the host cricoid cartilage of young or adult rabbits (Paper IV). Ki-67 was weakly expressed in the proliferative layer of cricoid perichondrium of young rabbits at one week after surgery. A similar expression of Ki-67 was found in the newly formed cartilage cells one week and four weeks after surgery in both age groups.

The proliferative layer of the cricoid perichondrium was thicker in young rabbits than in adult rabbits (Paper IV). rhBMP-2 induced a marked chondrogenesis in both experimental age groups. However, the newly formed cartilage was more elongated in young rabbits, and the segments of cricoid perichondrium that responded by chondrogenesis to the rhBMP-2 were longer in young rabbits than in adult rabbits at one week. The neochondrogenesis was located in adult rabbits more towards the tip of the cricoid cartilage cut end at one week.

Four weeks after surgery

The cricoid cartilage defect was completely repaired after four weeks by the formation of new bone and cartilage in rabbits treated with rhBMP-2, and the airway anatomy was undistorted. Chondrophyte (osteophyte) formation was not stimulated at the site of the cricoid cartilage defect in rabbits treated with rhBMP-2. The mucosa was completely covered in all rabbits by an epithelial layer after four weeks (Paper I).

The shape of new bone tissue corresponded to the shape of the inserted CS. There were no discontinuities or gaps at the margins of the cartilage defects (Paper II).

Safranine O/fast green staining showed that PGs had been synthesized between the host cricoid cartilage and new bone four weeks after surgery (in the area of newly formed cartilage) (Paper II).

The general aspects of the vascular pattern, and the pattern of expression of TIMP-1, were similar in control rabbits and treated rabbits, both one week and four weeks after surgery (Paper II).

The new bone was prevalent in the repair tissue of the cricoid defect. Abundant bone marrow was present within trabecular bone. ALP was active in the bone trabeculae (Paper III).

The cell proliferation marker Ki-67 was strongly expressed only in the bone marrow of new bone tissue four weeks after surgery (Paper III).

Histomorphometric analysis of the newly formed cartilage showed that there were no significant differences between young and adult rabbits one week and four weeks after surgery (but these values were not numerically corrected for relative differences in cricoid lumen size and cricoid cartilage thickness in young and adult rabbits) (Paper IV). However, the neochondrification area was significantly larger at four weeks than it was at one week, in both young and adult rabbits. New bone, with well-defined trabeculae, was present in the central part of the cricoid cartilage defect four weeks after surgery in young and adult rabbits treated with rhBMP-2. Bone marrow was also present.

Host cricoid cartilage was not calcified in young rabbits, while the central region of the host cricoid cartilage in adult rabbits was extensively calcified. The central part of the cricoid defect area was extensively calcified at four weeks in all age groups, as expected, since new bone had grown (Paper IV).

Collagen type I was more strongly expressed in the fibrous layer of the host cricoid perichondrium of adult rabbits than the mild expression in the fibrous layer of the host cricoid perichondrium of young rabbits. Collagen type I was also present in the new bone, granulation (fibrous) tissue, and newly formed cartilage (at one week after surgery) in both age groups (Paper IV).

Collagen type X was strongly expressed in the lacunar (pericellular) space in the central region of the host cricoid cartilage of all adult rabbits. However, the host cricoid cartilage of young rabbits expressed collagen type X only four weeks after surgery. Newly formed cartilage expressed collagen type X one week and four weeks after surgery in both young and adult rabbits (Paper IV).

Collagen type II was diffusely expressed in the matrix of the host cricoid cartilage, the proliferative layer of cricoid perichondrium, and newly formed cartilage. The expression of collagen type II was similar in young rabbits and adult rabbits (Paper IV). New bone and new cartilage were not induced in control rabbits, and the defects were filled by fibrous scar tissue.

Ultrastructural data – rhBMP-2 treated specimens (Paper V)

One week after surgery

Light microscopy – semi-thin sections

Appositional cartilage growth was found at the site of cricoid defect. A residue of the collagen sponge was present, and consisted of numerous, irregularly arranged, collagen-fiber-like structures. Numerous immature fibroblast-like cells had infiltrated the collagen sponge. Mineralized extracellular matrix and osteoblasts were seen close to the margin of newly formed cartilage. Osteocytes were enclosed in the mineralized matrix.

Electron microscopy – ultra-thin sections

Newly formed cartilage had a well-defined extracellular matrix consisting of collagen type II (small thin fibers) and proteoglycans (small dense particles).

Active fibroblast-like cells had infiltrated the implanted collagen sponge. These cells had started to form an immature matrix of collagen type I (thick fibers scattered in random directions).

Mineralized collagen type I (bone) matrix was present at the boundary between the newly formed cartilage and the residual collagen sponge. Some fibroblast-like cells had differentiated and appeared as osteoblasts well attached to the fibers of residual collagen sponge. The osteoblasts had large eccentrically located nuclei and cytoplasm that contained many mitochondria and an abundance of granular endoplasmic reticulum, indicating high activity. The osteoblasts were located close to the newly formed cartilage, in the area where mineralized collagen type I matrix had formed. Some of the osteoblasts, such as osteocytes with a well-defined pericellular zone (lacuna) and cytoplasmic processes, were trapped into the mineralized matrix.

Multinucleate cells resembling osteoclasts were also present, attached to fibres of the residual sponge. The osteoclast-like cells were large, had numerous mitochondria, and expressed an immature ruffled border in the area of contact with fibers of the residual collagen sponge.

Two weeks after surgery

Light microscopy – semi-thin sections

Mineralized osteoid-like matrix had formed at the site of the cricoid defect. Some small osteocytes were present in the mineralized osteoid matrix. Many osteoblasts were present on the surface of the osteoid matrices. There were many invading blood vessels at the boundary between newly formed bone matrix and cartilage.

The area of regenerated cartilage was greater two weeks after surgery than it was one week after surgery. Many proliferating chondrocytes were present.

Electron microscopy – ultra-thin sections

The distal part of the newly formed cartilage matrix (collagen type II) had become mineralized. Chondrocytes were hypertrophied in this region. The hypertrophic chondrocytes had enlarged, vacuolated nuclei, with accumulations of particulate glycogen and lipid droplets in the cytoplasm. Many newly formed blood vessels invaded the mineralized cartilage matrix, together with many osteoblasts, generating layers of osteoid matrix. Furthermore, the number of osteocytes was higher, not only in the proximity of newly formed cartilage but also in the central part of cricoid defects.

A mixture of unmineralized collagen type I, unmineralized collagen type II, and proteoglycans was present in some areas at the boundary between the newly formed cartilage and the newly formed bone matrix.

Many multinucleate cells were present in the area of cricoid cartilage defects two weeks after surgery. The multinucleate cells close to the newly formed cartilage were similar to chondroclasts. They were attached to mineralized cartilage matrix and expressed a thin and poorly structured (immature) ruffled border. The osteoclasts – another type of multinucleate cells – were attached to mineralized osteoid matrix and expressed a well defined clear zone and ruffled border.

Some dying (necrotic) cells were present in the vicinity of the newly formed blood vessels.

Four weeks after surgery

Light microscopy – semi-thin sections

The mineralized osteoid matrix had been replaced by trabecular bone containing osteocytes. Many osteoblasts were arranged on the surface of the bone trabeculas. Adipocyte-rich bone marrow and various myelogenic cells were present within the trabecular bone.

The mineralized cartilage matrix had been invaded by blood vessels. The columnar structure of the newly formed cartilage was well-defined, and it had a morphology that was similar to that of epiphyseal growth plates.

Electron microscopy – ultra-thin sections
Osteocytes surrounded by mineralized collagen I matrix (bone trabeculas) were present over the complete area of the cricoid defect. Osteoblasts, and occasional osteoclasts, covered the surfaces of bone trabeculas. The osteoclasts had prominent clear zone and ruffled borders showing full activity.

Newly formed cartilage consisted of columns of chondrocytes at different stages of differentiation, and mineralized cartilage lacunae invaded by capillaries and osteoblasts. The chondroclasts were also present in this region.

We found some dying (necrotic) cells in some areas.

Discussion

Qualitative and quantitative characteristics of repair tissue
The host cartilage and newly formed cartilage are poorly integrated following surgical treatment. The components of the newly formed (repair) cartilage and the host cartilage do not bind well to each other, and the cartilage structures of the organs involved do not function properly.

Sellers et al.[53] found discontinuities and gaps at the edges of articular cartilage defects in 22% of cases in rabbits treated with rhBMP-2. Shapiro et al.[1] suggested that the gaps allow micromotion and macromotion between the repair cartilage and the host cartilage, and that this motion initiates cartilage degeneration.

It is difficult to achieve good cartilage regeneration in a clinical context when treating different types of inflammatory arthritis with rhBMP-2. BMP-2 enhances chondrocyte proteoglycan synthesis only in the absence of interleukin 1alpha[2].

We have treated cricoid cartilage defects with rhBMP-2 delivered on a collagen sponge. The repair tissue that formed contained new bone and cartilage four weeks after surgery. The repair tissue adhered to, and was well integrated with, the host cartilage. We conclude that rhBMP-2 improves the repair of airway cartilage more than it improves the repair of articular cartilage.

Cartilage PGs play a major role in degenerative and inflammatory joint diseases, and they have been extensively studied because of this role. The loss of PGs is an early event in the destruction of the articular cartilage, and this loss is associated with a decreased ability to resist compression under load[3]. Sellers et al.[52, 53] used rhBMP-2 to heal full-thickness defects of articular cartilage in rabbits. They showed that rhBMP-2 greatly accelerates the formation of new subchondral bone and improves the overlying articular cartilage surface. They also showed that PGs are present in newly formed articular cartilage as early as four weeks after surgery, which agrees with our results.

The level of PG synthesis was also raised in articular cartilage in mice after rhBMP-2 injection into the knee joint[55].

Osteogenesis was strictly limited to the inserted collagen sponge and no new bone formed around the cricoid defect area. Abundant bone marrow was observed within trabecular bone.

Bessho et al.[4] showed that new bone rich in bone marrow is induced in rat muscles after the implantation of an rhBMP-2-collagen complex. They suggested that bone tissue induced by rhBMP 2 receives a continuous blood supply and has the potential to become self-supporting bone. This bone is continuously remodeled and its form is maintained over long periods.

Cui et al.[5] have presented similar results in rabbit. They treated a thyroid cartilage defect measuring 5 x 6 mm^2 with 5 mg of bovine bone ceramic combined with 1 mg of bovine BMP. The amount of new bone increased during the interval from 4 to 12 weeks, and the defects were completely filled with new bone after 16 weeks.

Okamoto et al.[6,7] treated tracheomalacia with BMP-2 delivered on gelatin sponges in dogs. They created a gap with dimensions 1 x 5 cm^2 in the anterior part of the trachea by removing ten sequential strips of cartilage of length 5 cm from the center of the cervical region. The tracheal mucosa was carefully preserved throughout the procedure. A gelatin sponge soaked in a solution containing 12 mg BMP-2 was implanted to replace the resected portion of the tracheal cartilage. Newly formed cartilage was present one month after implantation of the sponge. Regenerated cartilage was induced at the ends of the stumps of the host cartilage, with the direction of regeneration towards the luminal or contra-luminal side, and not towards the center of the gap in the cartilage. However, the regeneration process stopped 3 months after implantation. The authors did not succeed in inducing any new ectopic bone at the sites

of implantation of the gelatin sponges soaked with BMP-2 solution.

The spatial pattern of chondrogenesis described by Okamoto et al. was similar to ours. However, they did not observe any new bone formation and the gap was filled with fibrous tissue.

Biomineralization, or biological calcification, is a regulated process that leads to the accumulation of large amounts of calcium phosphate [8]. Calcium deposition is directly related to bone formation and hypertrophic chondrocytes[9]. In addition, ALP, a membrane-bound metalloenzyme that catalyses the hydrolysis of phosphomonoesters at an alkaline pH, is required for the mineralization of bone and cartilage[10].

ALP activity was present one week after surgery in the newly formed cartilage. Moreover, alizarin red S staining revealed irregularly shaped calcified matrix at the margin of the newly formed cartilage. These findings suggest that new chondrocytes support bone formation at the site of the cricoid cartilage defect.

Nakagawa et al. [11] studied the development of ectopic bone induced by a BMP collagen complex within muscle tissue. They showed that small amounts of irregularly shaped calcified tissue were present at the rim of the collagen pellet from day 10 after implantation, and that ALP was active inside the pellet from day seven after implantation.
A side-effect of BMP-2 in articular cartilage is that the formation of chondrophytes is stimulated[12]. Airway anatomy was not distorted in our model, nor was chondrophyte (osteophyte) formation stimulated.

The origin of repair tissue

Perichondrium forms new cartilage in animal models, showing that it has chondrogenic potential[13, 14, 15]. Different amounts of new cartilage formed in these studies, and the amount was small in all of them.

We have evaluated the role of the cricoid perichondrium in the repair of cricoid cartilage defects treated with rhBMP-2 in rabbits. The perichondrial flaps contained both inner (proliferative) and outer (fibrous) layers. Chondrogenesis was not induced by the perichondrial flaps in control rabbits or in rabbits treated with rhBMP-2, nor was repair cartilage induced at the sites of cricoid cartilage where the perichondrium was removed. These results agree with the findings of Duynstee et al.[9] It is, however, possible that the inner layer of the cricoid perichondrium was partly destroyed during perichondrial flap preparation, thereby reducing the capacity of the perichondrium for cartilage genesis.

In contrast, cartilage growth was induced from cricoid perichondrium that was left intact in rabbits treated with rhBMP-2, showing that newly formed cartilage originates from progenitor cells of the host perichondrium of cricoid cartilage.

New bone had formed in all rabbits treated with rhBMP-2 four weeks after surgery.

There is a large reservoir of cells in the body capable of osteogenesis throughout life[16]. We have shown that BMPRs, and proliferation cell nuclear antigen are moderately expressed in muscles adjacent to the cricoid, and this suggests that new bone may originate from skeletal muscles that are adjacent to the cricoid cartilage. This is logical, since muscle is the main tissue surrounding the cricoid cartilage.

It is more difficult to determine which cell type in the muscle is the origin of new bone. Previous work has suggested that satellite muscle cells are the source of new bone[17].

Skeletal muscle satellite cells are mononucleated cells, located between the sarcolemma and the basement membrane of terminally differentiated muscle fibers[18]. These cells are normally inactive in adult muscle, and act as a reserve population of cells that are able to proliferate in response to injury, giving rise to regenerated muscle and to more satellite cells. However, not all satellite cells are identical, and the regenerative compartment of skeletal muscle may contain subsets of precursor cells that differ in their phenotype and in their function.

Asakura et al. [19] explored the osteogenic potential and the adipogenic potential of satellite cells isolated from adult mice. Their experiments showed clearly that muscle satellite cells possess a multipotential activity that is characteristic of mesenchymal stem cells, and that they are capable of forming osteocytes, adipocytes, and myocytes.

Skeletal muscle tissue consists predominantly of myofibres and satellite cells, but it contains a wide range of other cell types, including connective tissue, fat, nerves, supporting cells, an extensive vascular system, and blood. It is thus possible that additional

sources of cells capable of osteogenesis are present in skeletal muscle.

It is also possible that adult skeletal muscle tissue contains a niche with a resident population of multipotent stem cells[20].

Vessel-associated stem cells may be resident or they may be derived from the circulation. It has been suggested that the multipotent stem cells isolated from various adult tissues are an equivalent population that seeds throughout the body, possibly during embryogenesis or via the circulation[21].

Age-related differences in cartilage repair and regeneration

It is necessary to consider both the rate of chondrogenesis and the qualitative pattern and local distribution pattern of chondrogenesis when assessing the healing of the airway cartilage in order to increase our understanding of the cellular mechanisms of this process.

We have shown that the proliferative layer of perichondrium is thicker in young rabbits than it is in adult rabbits, while the fibrous layer is thinner. We have also shown that a cell proliferation marker is expressed weakly in the perichondrium of young rabbits one week after surgery, and that it is not expressed in adult rabbits. Furthermore, the segments of cricoid perichondrium that responded to the rhBMP-2 were longer in young rabbits.

The toluidine blue staining was more intense in discrete regions in newly formed cartilage of young rabbits. Moreover, the cell-matrix ratio of newly formed cartilage differed between young and adult rabbits, with matrix prevailing in adult rabbits.

Thus the histological pattern of cartilage formation differed qualitatively in young and adult rabbits, while that of bone formation was similar. It appears that the final osteogenesis has not been affected by the differences in the pattern of appositional cartilage growth between young and adult rabbits.

The repair of defects of cricoid cartilage induced by rhBMP-2 may proceed by a mechanism in which angiogenesis is partially inhibited. This would give a prevalence of chondrogenesis. We speculate that in such a case the amount of newly formed cartilage induced in young rabbits is much higher than in adult rabbits.

The morphometric measurements show that the appositional growth of cartilage in adult rabbits follows a ”catch-up” pattern. Growth is initially slow, but by four weeks after surgery, the area of newly formed cartilage in adult rabbits has reached the value found in young rabbits.

Young rabbits have a smaller airway lumen than adult rabbits. The elongated form of chondrogenesis in young rabbits may have a functional importance, in that it avoids narrowing the airway lumen. Moreover, the pattern of chondrogenesis, natural or induced, may determine the critical size of an airway cartilage defect that heals properly.

Verwoerd-Verhoef et al.[22] studied the growth dynamics of the cricoid cartilage in young rabbits, and showed that the mitotic activity of cricoid chondrocytes is restricted mainly to the first 4 weeks of life. We have shown in the work reported here that a cell proliferation marker is not expressed in host cricoid cartilage during this period.

Wagner et al.[23] demonstrated that the (scar-free) neonatal regenerative healing of rat cartilage takes place by proliferation of the resident already-differentiated chondrocytes (interstitial growth). We have shown that rhBMP-2 can only induce the appositional growth of cricoid cartilage by the proliferation of progenitor cells from the perichondrium, and the subsequent differentiation of these cells into chondrocytes (in both age groups). It appears that the mature chondrocytes of the cricoid rarely proliferate and cannot form the basis for an effective regeneration of cricoid defects.

Mankarious et al.[5] studied the expression of collagen type I in human tracheal cartilage of different ages (2, 4, 11, 15, 39 years old). They found collagen type I staining within the lacunae and extracellular matrix, and in the perichondrium. Naumann et al.[2] showed that collagen type I is expressed within the lacunae of rabbit hyaline cartilage of the nasal septum. We have shown that collagen type I is expressed only in the fibrous layer of the perichondrium of cricoid cartilage of young and adult rabbits. Collagen type I is more strongly expressed in the perichondrium of adult rabbits than it is in the perichondrium of young rabbits.

Mankarious and Goetinck[24] showed that collagen type X is not expressed in human cricoid cartilage at one year and four years of age. However, they showed that collagen type X is weakly expressed in cricoid cartilage specimens of a 13-year-old. The cricoid cartilage of young rabbits expressed collagen type X four weeks after surgery only; not one week after surgery. However, collagen type X was strongly expressed in cricoid cartilage of adult rabbits. The latter observation is consistent with the strong calcification of the cricoid

cartilage of adult rabbits, because collagen type X is associated mainly with calcified and hypertrophic cartilage.

We have shown that collagen type X is expressed in the newly formed cartilage of both age groups. It appears that newly formed cartilage supports the primary calcification of the extracellular matrix, found at the margin of implanted collagen sponge one week after surgery.

The results described above lead us to suggest that the same dose or a lower dose of BMP-2 may be used to treat defects of airway cartilage in children than the dose used for adults.

Ultrastructural characteristics of repair tissue

Local treatment of cricoid cartilage defects with rhBMP-2 delivered on an absorbable collagen sponge triggered an osteogenic process that closely resembles embryonic osteogenesis (Figure 4).

A primary ossification center initially formed at the boundary between newly formed cartilage and the residual collagen sponge. This process had already started one week after surgery. The fibroblast-like cells that were present then proliferated into a condensed and compact structure, and later differentiated into osteoblasts. The osteoblasts formed a new collagen type I matrix that later became mineralized. The process is similar to the intramembranous bone formation described by Riminucci et al.[1] in the bony collars of endochondral bones of rat fetuses. The initial deposition of bone in the rat fetus requires the formation, ex novo, of a mineralized structure in a non-mineralized tissue. Furthermore, the priming of bone deposition in the rat fetus involves hypertrophic chondrocytes.

The intramembranous bone formation in our experimental model had started one week after surgery, and it was followed by, and combined with, the formation of endochondral bone. The markers of

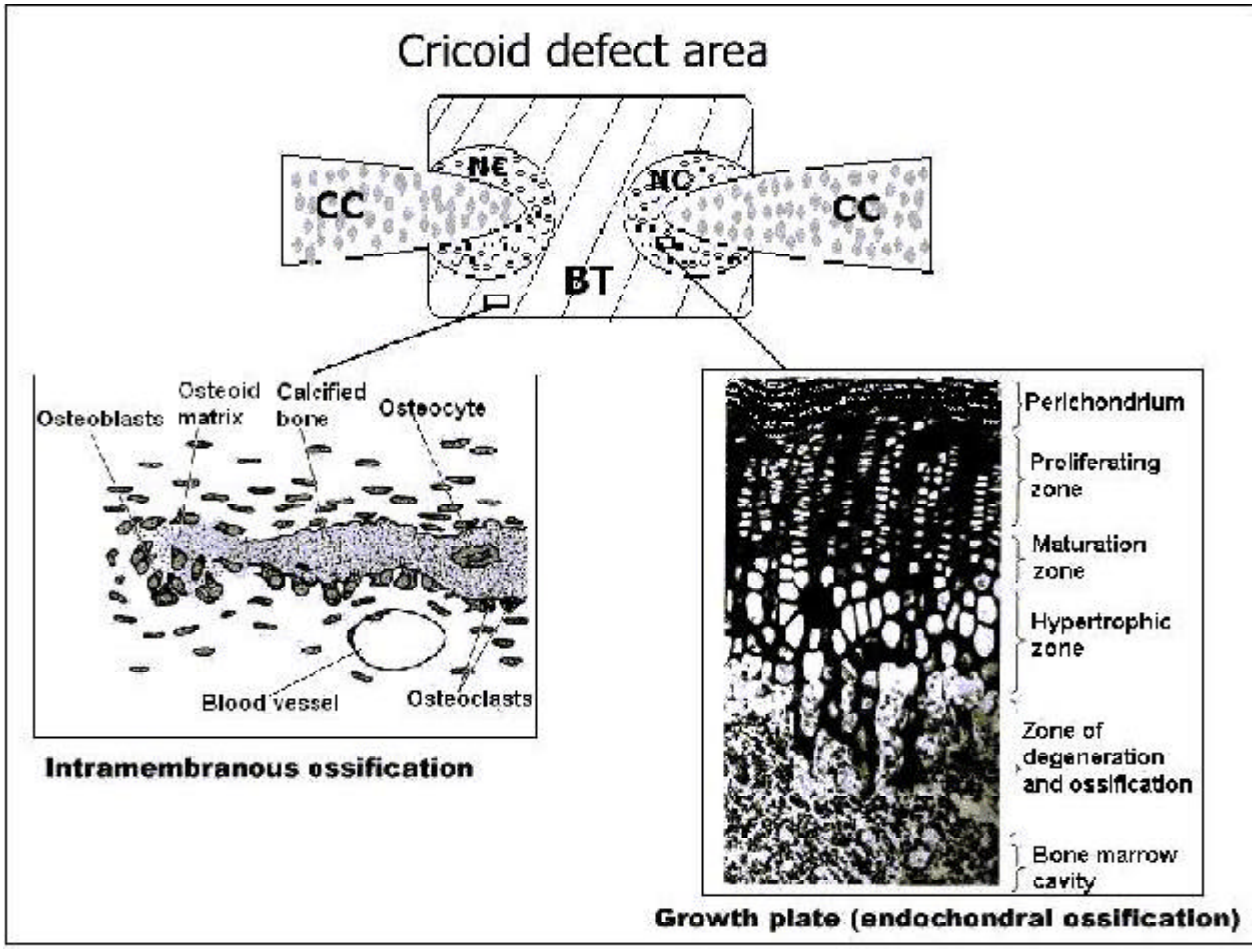

Figure 4. Schematic diagram of the ossification process that occurs at the site of cricoid cartilage defects treated with rhBMP-2. CC – cricoid cartilage ends; NC – newly formed cartilage; BT – bone tissue.

endochondral osteogenesis (the invasion of the mineralized cartilage matrix by newly formed blood vessels and the presence of osteoblasts that deposit collagen type I onto a pre-existing cartilage matrix) were present at the site of cricoid cartilage defects two weeks after surgery.

Nakagawa et al.[63, 64] studied the ultrastructural characteristics of the ectopic formation of bone induced by a BMP collagen complex in rat muscles. They showed that the BMP collagen complex can induce the formation of new bone both directly, without cartilage being formed, and indirectly, through an intermediary chondroid scaffold. They also showed that chondroclast-like cells are present, as is calcified cartilage matrix, 14 days after rhBMP-2 implantation.

We have shown that chondroclasts are attached mainly to the mineralized cartilage matrix two weeks and four weeks after surgery. These chondroclasts are similar to those described by Nordahl et al.[2] Chondroclasts do not form ruffled borders and clear zones to the same extent as osteoclasts. Instead, chondroclasts tend to express an undifferentiated surface adjacent to the matrix – a surface that is structurally the same as the plasma membrane. The differences between chondroclasts and osteoclasts may reflect the maturation steps of chondroclasts as they develop into osteoclasts. Osteoclasts with fully developed ruffled borders and clear zones are present two weeks after surgery and are numerous four weeks after surgery. This shows that the bone matrix is remodeled, and we interpret our observations as the maturation process of bone.

Activation of clasts (which involves the development of a well-defined ruffled border and clear zone) is promoted by interactions between integrins at the plasma membrane of clasts and extracellular proteins such as osteopontin (OPN)[3,4]. OPN is produced mainly by osteoblasts close to osteoclasts in vivo, and it is produced by osteoblasts later in the differentiation than other bone matrix proteins, such as bone sialoprotein[5]. Most of the clasts that we observed had chondroclast morphology, which may reflect the low concentration of activation proteins such as OPN, given that the processes we are studying are the early stages of bone formation.

Moreover, it appears that the rate of matrix mineralization contributes to of the development of the phenotype of chondroclasts and osteoclasts. Nordahl et al.[6] showed that the ruffled borders and clear zones of osteoclasts and chondroclasts are profoundly altered in favor of a less well defined intermediate zone in vitamin D deficiency rickets, and they showed that poorly mineralized tissue is expressed in this condition.

Irie et al.[7] examined osteoclast differentiation in the process of ectopic bone formation in rats, induced subcutaneously by rhBMP-2. Osteoclasts at various stages of development, classified by the pattern of immunoreactivity for cathepsin K, were present seven days after rhBMP-2 implantation, before bone matrix had formed. We have also shown that a few multinucleate cells similar to osteoclasts are present as early as one week after surgery. These multinucleate cells were attached to the fibers of the residual collagen sponge, and were probably involved in collagen sponge resorption.

Palumbo et al.[8] studied apoptosis in the intramembranous perichondral centres of ossification of various long bones in newborn rabbits. They showed that apoptosis affects mesenchymal cells that are located between the forming trabeculae and capillaries, which suggests that apoptosis during bone formation makes space available for the advancing bone growth. We also found some dying (necrotic) cells located in the vicinity of newly formed blood vessels two weeks, and four weeks, after surgery.

Interestingly, the local treatment of cricoid cartilage defects with rhBMP 2 induces the formation of cartilage that has a morphology that resembles that of growth plates, a morphology that has been observed only in long bone epiphysis. The growth plate like structure derives from the progenitor cells of the perichondrium after direct stimulation with rhBMP-2.

Wozney[24] has shown that the relative amounts of endochondral and intramembranous ossification induced by rhBMP-2 depend on the concentration of BMP, the site of implantation, and the nature of the carrier material. Moreover, no stable (persistent) cartilage formation takes place at any BMP concentration in experiments in which the BMP has been implanted intramuscularly or subcutaneously. The cartilage has always been replaced by bone in such experiments.

This is also the case in our experiments. The sole role of newly formed cartilage at the site of cricoid defects is that of acting as a functional support for new bone formation.

Hoffmann et al.[47] suggested that BMPs are involved in the induction of chondrogenic development, rather

than its progression. Several other signaling cascades regulate and promote chondrogenesis. Our results support this suggestion. It appears that rhBMP-2 alone cannot restore cricoid cartilage defects with tissue of a similar type (cartilage). Cricoid cartilage defects are instead repaired by new bone tissue.

The results of the present study show that local progenitor cells and growth factors, in combination with microsurgical techniques, offer new methods for treating airway stenosis, deformities or trauma. BMP-2, however, is not sufficient to treat large defects of the airway. Future studies will focus on developing carrier materials that have mechanical properties and surgical practicality appropriate for the controlled release of rhBMP-2. Moreover, a combination of rhBMP-2 with other growth factors (such as fibroblast growth factor-2) may be considered.

Conclusions

rhBMP-2, delivered on an absorbable collagen sponge, induces the regeneration and the repair of rabbit cricoid cartilage defects. It induces a faster relining and regeneration of airway epithelium than those that occur in the absence of rhBMP-2.

The repair tissue induced by rhBMP-2 consists of new bone and cartilage integrated with the host tissue at the site of the cricoid cartilage defects. Moreover, the newly formed cartilage matures and produces proteoglycans.

The perichondrium of cricoid cartilage is a potent source of progenitor cells. Repair cartilage tissue appears to originate only from the progenitor cells of the host perichondrium that adheres to cricoid cartilage in rabbits treated with rhBMP-2. New bone may originate from local skeletal muscle, and newly formed chondrocytes support bone formation at the site of the cricoid cartilage defect.

rhBMP-2 induces temporal and quantitative patterns of new bone formation at the site of cricoid cartilage defects that are similar in young and in adult rabbits. Chondrogenesis, in contrast, is qualitatively different in young rabbits from that in old rabbits. Appositional cartilage growth is more easily induced from the cricoid perichondrium of young rabbits than from the cricoid perichondrium of adult rabbits.

Intramembranous osteogenesis and endochondral osteogenesis take place at the site of cricoid cartilage defects treated with rhBMP-2 in rabbits. Endochondral osteogenesis follows intramembranous osteogenesis.

Progenitor cells from the host perichondrium of the cricoid cartilage can form, when stimulated by rhBMP-2, a growth plate-like structure that is similar to the epiphyseal growth plate.

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