The development, structure and repair of articular cartilage

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Functional barrier principle for growth-factor-based articular cartilage repair

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Summary

Objective: Induction of growth-factor-based repair in full-thickness articular cartilage defects can be impaired by the upgrowth of blood vessels and new bone into the cartilaginous compartment. We postulated that if an antiangiogenic factor (suramin) is included in the chondrogenic matrix applied to the cartilaginous compartment of a full-thickness defect, vascular upgrowth and therefore bone formation will be inhibited (functional barrier principle).

Design: Full-thickness defects were created in miniature pigs and the bony portion filled with a chondrogenic matrix. The cartilaginous compartment was filled with the same matrix which additionally contained suramin, either in a free form or in free and liposome-encapsulated forms. Animals were sacrificed 8 weeks after surgery and the extent to which bone tissue had encroached on the cartilaginous compartment was graded semiquantitatively using light microscopy.

Results: In 63% of the control defects, bone represented more than 50% of the repair tissue present. In 10% of the defects treated with free suramin, bone upgrowth was completely inhibited; in 55%, osseous tissue occupied 1–10% of the cartilaginous space and in the other 35%, it represented 11–50% of the repair tissue present in this compartment. In 69% of the defects treated with free and liposome-encapsulated suramin, bone upgrowth into the cartilaginous compartment was completely inhibited; in the remaining 31%, osseous tissue occupied no more than 1–10% of this space.

Conclusions: To be effective, an antiangiogenic factor needs to be present at a sustained level throughout the chondrogenic treatment course.

Abbreviations: BMP-2, bone morphogenetic protein-2, IGF-1, insulin-like growth factor-1, TGF-β1, transforming growth factor-beta 1.

Introduction

A growth-factor-based approach to the induction of articular cartilage repair has the advantage of avoiding cell or tissue transplantation[1]. The availability of such strategies permits the surgeon to act instantaneously on a decision to treat as determined during exploratory surgery or arthroscopy[2,3]. Using a small, partial-thickness articular cartilage defect model, the biologic obstacles that must be overcome for successful repair have been delineated[1,4]. Basically, the lesion must be filled with a biodegradable matrix containing a free chemotactic and mitogenic factor to stimulate the migration of potential repair cells from the synovial pool and their subsequent proliferation, and with a liposome-encapsulated chondrogenic agent[5] to induce a timely transdifferentiation of primitive repair cells into chondrocytes, which then lay down cartilage.

Although this strategy works well for partial-thickness defects, it cannot be applied to full-thickness lesions without additional considerations being taken into account[5]. This latter type of defect penetrates the subchondral bone and bone-marrow spaces wherein reside an abundance of different cell types[6–8] (such as pericytes, perivascular mesenchymal stem cells, hemopoietic cells, blood cells, vascular endothelial cells and fat cells), some of which are not only responsive to substances in the implanted construct[9–11], but respond in such a manner as to trigger the onset of undesired angiogenic and osteogenic processes. After stimulation by growth factors, blood vessels grow rapidly from the bony floor of the defect and invade the cartilaginous compartment so swiftly that the biologic conditions soon become unfavorable to chondrogenesis and conducive to osteogenesis[12]. By inserting a cell-excluding membrane (or structural barrier[13,14]) at the cartilage–bone interface, angiogenic activities and the dependent osteogenic processes can be physically prevented from encroaching on the cartilaginous compartment[12]. However, optimal surgical placement of such a membrane at the appropriate level cannot be readily achieved by macroscopic inspection, nor is it easy to seal it completely at the defect edges by press-fitting. Consequently, not only does the cartilage–bone interface undulate in height, but osseous tissue upgrowth occurs through microscopic chinks between the membrane and defect walls. Moreover, the long-term persistence of poorly biodegradable membranes can prevent repair cartilage from becoming firmly anchored to the underlying bone[15,16], as can mild inflammatory responses triggered by slowly released degradation products. The attachment of such ill-seated tissue is liable to become additionally weakened with time owing to the action of shear forces during normal joint usage[17].

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An alternative therapeutic approach would be to apply a functional barrier, such as an antiangiogenic substance, which would chemically impede rather than physically block vascular upgrowth into the cartilaginous compartment.

It was the aim of the current study to test the efficacy of such a principle in achieving compartment-specific repair tissue formation while avoiding the problems associated with a structural barrier. The authors used a small, full-thickness defect model in Goettingen miniature pigs[12]. Lesions were filled with the same chondrogenic matrix that was used to test the structural barrier principle[12]. However, the construct implanted in the cartilaginous compartment also contained the antiangiogenic factor suramin[18,19]. This treatment strategy substantially impeded the upgrowth of vessels and bone into the cartilaginous region, thereby favoring a compartment-specific repair tissue result.

Materials and methods

SURGICAL PROCEDURE

Eight adult Goettingen miniature pigs (2–4 years of age) were used for this study. General anesthesia was induced by an intravenous injection of Narketan® (Chassot AG, Belp, Switzerland) and maintained by administering Halothan® (Halocarbon Laboratories, River Edge, NJ) and nitrous oxide. The knee of each hind limb was exposed and four full-thickness defects (approximately 0.8 mm in depth×1.2 mm in width×7–8 mm in length) were created through the articular cartilage layer of the trochlear groove (two defects on the medial and two on the lateral facet) using a custom-built planing instrument[4]. One (five animals) or two (three animals) additional defects were created in the medial femoral condyle of each knee. Eighty-six defects were generated in the eight animals. These were blotted dry and filled with an aqueous solution of chondroitinase ABC (1 unit/ml [Sigma–Aldrich, Buchs, Switzerland]), which was removed by swabbing after 4–5 min. Each defect was rinsed thoroughly with physiologic saline and again blotted dry[12].

In six of the miniature pigs, defects received a matrix composed of gelatin (Gelfoam®, Upjohn Company, Michigan, MI) and fibrin (purified from bovine blood according to the method described by Mosher and Blout[20]). In the other two animals, defects received a matrix composed of fibrin alone. The latter served as controls to exclude the possibility of a gelatin-specific effect.

The matrix composed of fibrin alone was prepared as a solution of fibrinogen (19 mg/ml of 0.15 mol Tris-buffered saline [Merck, Dusseldorf, Germany]) to which human thrombin (1 unit/ml [Immuno, Vienna, Austria]) was added shortly before introduction into the defect. The matrix composed of fibrin and gelatin was prepared as a solution of fibrinogen (19 mg/ml) and gelatin (0.2 g/ml of 0.15 mol Tris-buffered saline) to which human thrombin (1 unit/ml) was added shortly before introduction into the defect.

Each matrix contained a free chemotactic and mitogenic factor, either TGF-β1 (4 ng/ml, porcine platelet [R&D Systems Inc., Minneapolis, MN]) or IGF-1 (10 ng/ml, human recombinant [Gibco Technologies, Basel, Switzerland]), and a liposome-encapsulated differentiation agent, either TGF-β1 (600 ng/ml) or BMP-2 (1 µg/ml [Genetics Institute, Cambridge, MA]). Liposomes were prepared and drugs incorporated according to the method described by Kim *et al.*[21].

The bony compartment of each defect was filled with one of the matrix mixtures (Table I) and allowed to polymerize *in situ*. The cartilaginous compartment of the same defect was filled with the same matrix containing the same combination of chemotactic factor and differentiation agent but additionally containing the antiangiogenic drug suramin (Germanin®, Bayer AG, Leverkusen, Germany [Fig. 1]). Suramin was present either solely in a free form (0.4 mol/l) or in free (0.4 mol/l) and liposome-encapsulated (0.4 mol/l) forms (Table I). The 25 defects containing mixtures without suramin (controls [Table I]) were distributed topographically such that 10 were next to other suramin controls and 15 were next to suramin-containing lesions. These two subgroups were evaluated separately to determine whether suramin had a local effect.

After *in-situ* polymerization of the matrix in the cartilaginous compartment, wounds were closed layer by layer. Postoperatively, the animals were permitted free cage movement until sacrifice 8 weeks later. Miniature pigs were sacrificed by administering a lethal dose of potassium chloride (to induce cardiac arrest).

TISSUE PROCESSING AND ANALYSIS

Hind limbs were amputated and the knees freed of soft tissues. The distal part of each femur was removed and sawed into three pieces (the upper half of the trochlear groove, the lower half of the trochlear groove, and the medial femoral condyle) using a diamond band-saw (Exact Medical Instruments, Oklahoma City, OK). For rapid histologic examination, one 1.5-mm-thick slice was cut from the lower half of the trochlear groove (parallel to the long axis), cryofixed using carbon dioxide, cryosectioned (in the same direction), and stained with toluidine blue O. The other tissue pieces were fixed by immersion in 2.5% (volume per volume) glutaraldehyde (Merck, Dusseldorf, Germany) and 2.5% (volume per volume) formaldehyde (Merck) buffered (pH 7.4) with 0.1 mol sodium cacodylate (Merck) for 2–4 days at ambient temperature. They were then dehydrated in ethanol and embedded in methylmethacrylate. Tissue slices 1.2 mm thick were sawed perpendicular to the longitudinal defect axis using a diamond saw (Leco, Warrendale, PA), glued onto polished Plexiglas object holders (Altumax France SA, Paris La Défense, France), milled to a thickness of approximately 100–150 µm with a Polycut E (Reichert-Jung, Heidelberg, Germany), polished, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin[1], and examined in a Vanox AH-2 light microscope (Olympus, Tokyo, Japan). Five to six tissue slices were produced along the length of each defect, three of these being randomly selected for semiquantitative histologic analysis (Table I).

Repair tissue laid down in the cartilaginous compartment of each defect was assessed qualitatively for its resemblance to native cartilage, with cell morphologic features and staining characteristics of the intercellular matrix being used as the relevant criteria. The percentage transformation of primitive mesenchymal repair tissue into cartilage-like tissue was estimated by point counting using a standard grid system[22]. In the bony compartment, osseous tissue was identified on the basis of its morphologic features and staining characteristics. The proportion of tissue represented by bone in the cartilaginous compartment was assessed semiquantitatively as falling into one of four categories: no bone formed (0%); 1–10% of the compartment's volume occupied by bone; 11–50% of the compartment's volume occupied by bone; and greater than 50% of the compartment's volume occupied by bone (Table I).

Table I
Treatment strategies and histologic findings

Composition of the chondrogenic matrix applied to the bony and cartilaginous compartments of each defect			Form of suramin in matrix applied to cartilaginous compartment	Total number of defects per experiment	Number and percentage (in parentheses) of sections with the indicated proportions of bone tissue in the cartilaginous compartment			
Matrix	Chemotactic and mitogenic factor (free form)	Differentiation factor (liposome-encapsulated)			0%	1–10%	11–50%	>50%
Gelatin and fibrin	TGF-β1	TGF-β1	No suramin	10	0 (0%)	0 (0%)	12 (40%)	18 (60%)
Gelatin and fibrin	IGF-1	BMP-2	No suramin	15	0 (0%)	0 (0%)	15 (33%)	30 (67%)
Gelatin and fibrin	TGF-β1	TGF-β1	Free	10	6 (20%)	15 (50%)	9 (30%)	0 (0%)
Gelatin and fibrin	IGF-1	BMP-2	Free	10	0 (0%)	18 (60%)	12 (40%)	0 (0%)
Gelatin and fibrin	TGF-β1	TGF-β1	Free and liposome-encapsulated	26	57 (73%)	21 (27%)	0 (0%)	0 (0%)
Gelatin and fibrin	IGF-1	BMP-2	Free and liposome-encapsulated	15	51 (65%)	27 (35%)	0 (0%)	0 (0%)

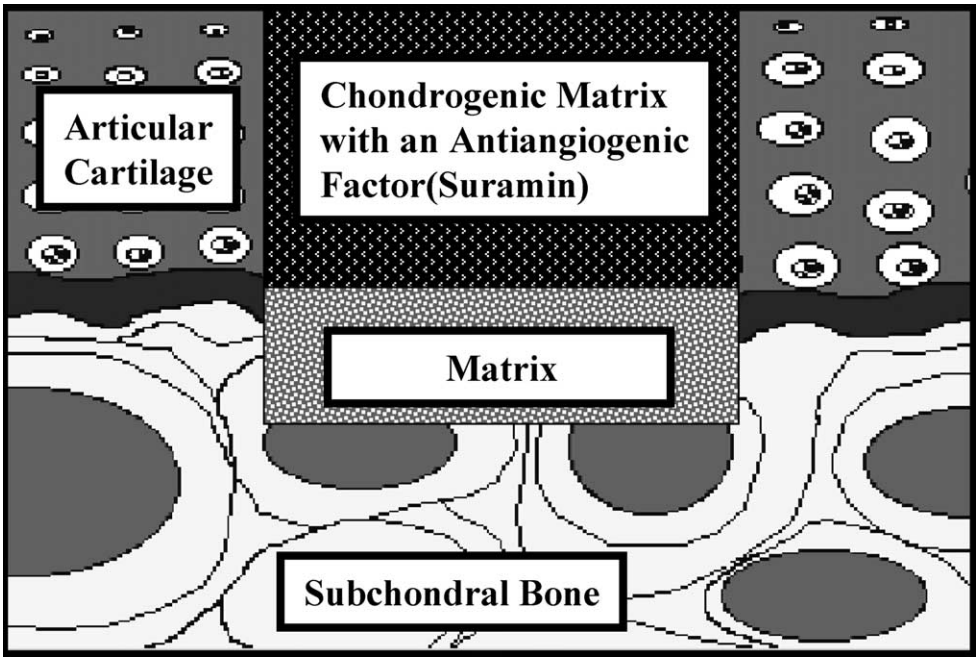

Fig. 1. The set-up used to test the functional barrier principle is shown. First the bony and then the cartilaginous compartment of a shallow, full-thickness defect were filled with a chondrogenic matrix. In the latter instance, the matrix contained the antiangiogenic factor suramin, whereas in the former, no such substance was included.

Results

Semiquantitative data pertaining to the experimental findings are summarized in Table I and graphically shown in Fig. 2. In control defects which had received no suramin and in which no antiangiogenic effect had been exerted, there was upgrowth of osseous tissue into the cartilaginous compartment in all cases. The degree of bone upgrowth into the cartilaginous compartment varied greatly, not only between different defects (Fig. 3) but also along the length of any particular lesion. Generally, however, the proportion of the repair tissue represented by bone within the cartilaginous compartment exceeded 50% (Fig. 2). No significant difference in this parameter was observed between control defects whose neighbors in the same joint were controls ($n=10$) and those which were neighbored by suramin-treated defects ($n=15$). This finding indicates that suramin had not leaked out of the defect to a significant degree. The distribution profile of numerical data was not influenced by the nature of the growth factor system used (free TGF-β1 and liposome-encapsulated TGF-β1 or free IGF-1 and liposome-encapsulated BMP-2).

In defects that had been treated with suramin in a free form alone (Fig. 4), upgrowth of osseous tissue into the cartilaginous compartment was only partially inhibited. As with controls, the degree of encroachment was variable; nevertheless, an obvious shift toward the lower-percentile categories was apparent, with a slight skew in favor of the 1–10% category being observed (Table I; Fig. 2). No more than 50% of the tissue laid down in the cartilaginous compartment of the defects was bone. The distribution profile of numerical data was not influenced by the nature of the growth factor system used. Most of the bone present was woven, which had been formed by direct ossification, not via the enchondral route.

In defects that had been treated with liposome-encapsulated and free suramin (Fig. 5), a dramatic suppression of osseous tissue upgrowth into the cartilaginous compartment was observed. In the majority of cases (69%), virtually no bone was present, and in no instance did it represent more than 10% of the total tissue volume (Table I; Fig. 2). The distribution profile of numerical data was not influenced by the nature of the growth factor system used.

The osseous compartment of each defect void was filled completely with highly vascularized woven bone (Figs. 3–5), bone marrow and adipose tissue. The mechanism of ossification could not be ascertained at the time of analysis (8 weeks postoperatively), bone formation being complete at this stage and remodeling activities well underway in some locations. Along the defect margins, repair and native tissue were well knit. That no differences between groups were observed in the bony compartment of defects indicates that suramin did not seep downward from the cartilaginous compartment to an appreciable degree.

The interface between repair cartilage and repair bone tissues generated in their respective compartments continued at the same level as that in the native surroundings, local deviations being no more than ±20%. These undulations were thought to reflect corresponding irregularities in the surface contour of the matrix introduced into the bony compartment during surgery. The two types of repair tissue were generally well knit in the plane of juxtaposition, which implies that suramin did not interfere with the integration process. The cartilage-like quality of tissue laid down in the cartilaginous compartment was similar using each of the two growth factor systems. The degree of transformation from mesenchymal tissue to cartilage-like tissue was in the range 50–80%, which corresponds to that found in a previous study[1]. This finding indicates that suramin had no influence on either the migration of cells from the synovium, their proliferation, or their differentiation into chondrocytes.

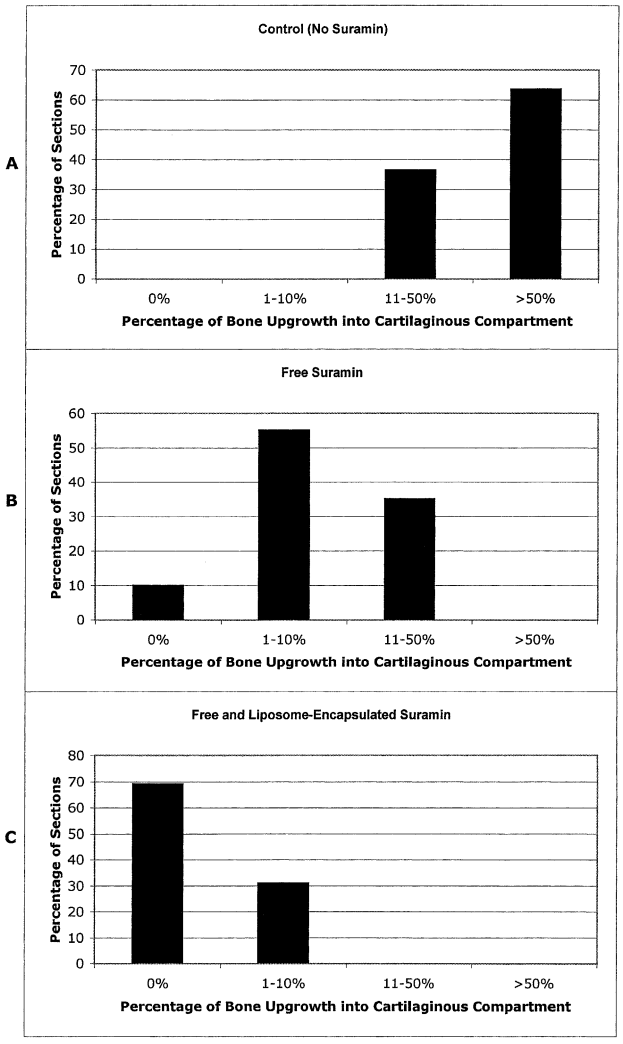

Fig. 2. (A) Semiquantitative histologic findings relating to control defects. In the absence of suramin, bone upgrowth into the cartilaginous compartment occurred in all defects; and in most instances (63%), osseous tissue represented more than 50% of the repair tissue present. (B) Semiquantitative histologic findings relating to defects whose cartilaginous compartment was treated with free suramin. In this group of defects, bone never represented more than 50% of the repair tissue present in the cartilaginous compartment; and in 65%, it was either absent altogether (10%) or occupied no more than 1–10% of this space (55%). (C) Semiquantitative histologic findings relating to defects whose cartilaginous compartment was treated with free and liposome-encapsulated suramin. In this group of defects, osseous tissue never occupied more than 10% of the repair tissue present in the cartilaginous compartment; and in 69%, bone upgrowth was completely inhibited.

Residues of gelatin were sometimes observed in the cartilaginous compartment of defects that had been treated with the composite matrix, whereas fibrin was always completely resorbed, irrespective of whether it had formed a part or the whole of an implant. Since gelatin is known to degrade less rapidly than fibrin, this finding is not suggestive of a suramin-related effect. In the bony compartment, no traces of gelatin were ever observed in any of the defects, indicating that its remodeling was more efficient in this well-vascularized region.

Discussion

The functional barrier principle (Fig. 1) arose from the need to inhibit vascular invasion of, and bone formation in, the cartilaginous compartment of full-thickness defects[12]. The success of this concept hinges on the premis that in the absence of blood vessels, osteogenesis cannot take place[23–26]. The angiogenic drug suramin[18,19,27] was chosen as the functional barrier compound.

The surgical procedure posed no difficulties. A composite matrix of gelatin and fibrin was selected for its good volume stability. Purely fibrinous implants are known to shrink uncontrollably during polymerization *in situ*[1], which can lead to poor integration between repair and native tissue along the defect floor and walls and to a concave articular surface. The non-suramin-containing matrix placed in the bony compartment could be leveled accurately by macroscopic inspection at the prospective cartilage–bone interface. The microscopic findings confirmed that the regenerated height of this junction corresponded to its level in the native surroundings. Local fluctuations did not exceed ±20% of the articular cartilage height (approximately 700 µm), which is within the limits of acceptability. It was considered best to slightly underfill the bony portion of the defect with the matrix lacking suramin and to overfill, to a corresponding degree in a downward direction, the cartilaginous compartment with the suramin-containing matrix [Fig. 5(C)]. Under these conditions, repair tissue laid down in each compartment will be remodeled and the natural level of the cartilage–bone interface eventually reestablished[6,7,28]. In the event of the osseous defect space being overfilled [Fig. 5(D)], it is unlikely that the bone tissue laid down above the natural border will be replaced by cartilage, because once vessels have invaded the cartilaginous region, the process cannot be reversed by any known physiologic or pathologic mechanism.

Eight weeks after surgery, osseous defect spaces were filled with well-vascularized woven bone, bone marrow and adipose tissue (Figs. 3–5), irrespective of the growth factor system used, indicating that osteogenesis had been stimulated efficiently. In some locations, remodeling into lamellar bone was already underway [Fig. 3(A)]. Repair and native bone tissue were well integrated along the lesion borders.

Suramin was only partially effective in suppressing bone tissue upgrowth into the cartilaginous defect space when present in a free form alone (Fig. 4), but highly efficacious when included in a delayed-release system (liposomes). In the latter case (Fig. 5), the cartilaginous compartment of most defects (69%) contained no bone, whereas in the remainder, it constituted maximally 10% of the repair tissue volume. These findings suggest that the half-life of freely dissolved suramin is short and that to be fully efficacious this compound must be present throughout the healing period.

Tissue transformation into cartilage within the cartilaginous compartment was approximately 50–80%, which corresponds to the range previously found using a partial-thickness defect model[1]. This finding shows that, at the concentration used, suramin neither inhibited the migration of cells from the synovium nor influenced their metabolic (remodeling) activity or capacity to differentiate into chondrocytes. Given the small volume of matrix deposited in the defects of each joint, and therefore the low total dose of suramin applied, no systemic effects were to be expected. In clinical practice, this compound is administered systemically at much higher levels in the treatment of sleeping sickness and onchocerciasis[29–31].

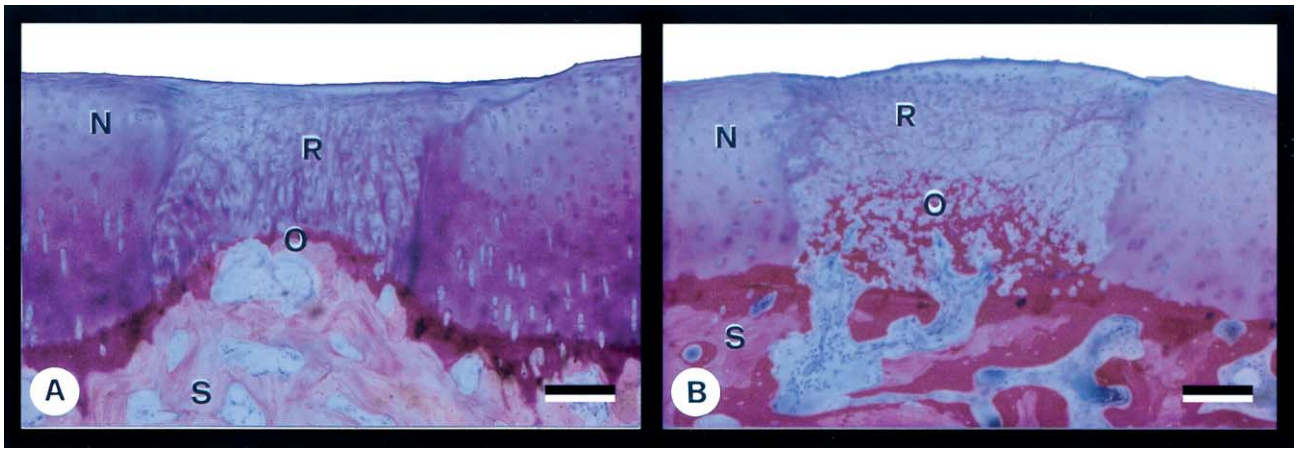

Fig. 3. (A) Light micrograph of a shallow, full-thickness defect 8 weeks after treating the entire lesion void with a chondrogenic matrix that contained no suramin. Osseous tissue (O) has grown upward from the subchondral bone region (S) and completely fills the bony compartment, of the defect. In the absence of an antiangiogenic factor (suramin), osseous tissue has grown unimpeded into the cartilaginous compartment where it constitutes approximately two-fifths of the repair tissue present. The remainder of the cartilaginous compartment contains a mixture of primitive mesenchymal and cartilage-like repair tissue (R). The partially differentiated cartilage-like tissue can be distinguished from native hyaline cartilage (N) by its higher numerical density of cells and coarser fibrillar nature. The newly formed osseous tissue has been remodeled into lamellar bone at most locations. An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=150 µm. (B) Light micrograph of a shallow, full-thickness defect 8 weeks after receiving the treatment described in (A). In this example, osseous tissue (O) occupies approximately one-half of the repair tissue laid down in the cartilaginous compartment. In contrast to (A), the newly formed osseous tissue has not yet been remodeled into lamellar bone but still has the morphologic features and staining characteristics of the woven type. S, subchondral bone; R, primitive mesenchymal and cartilage-like repair tissue; N, native hyaline cartilage. An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=150 µm.

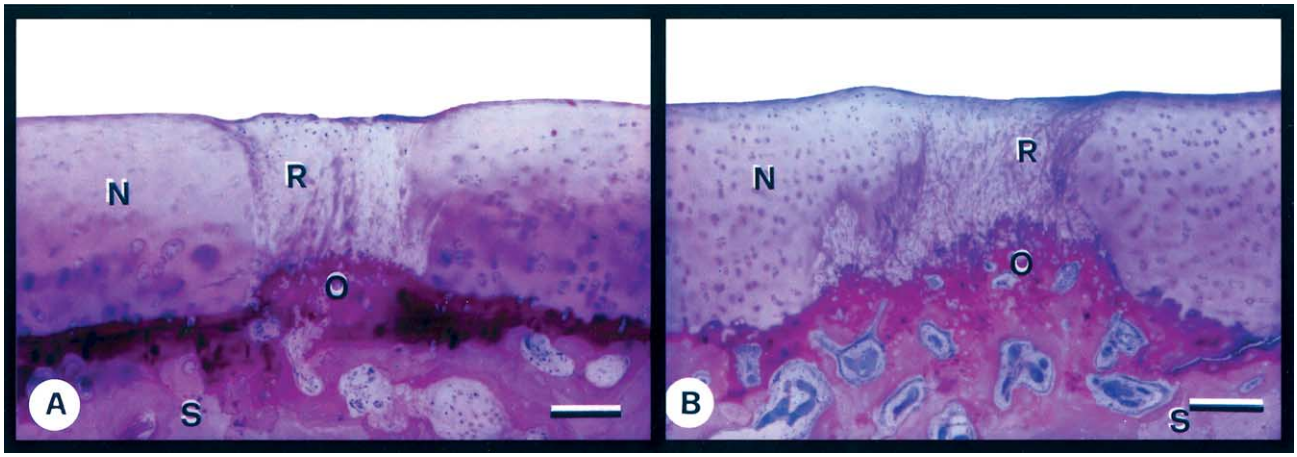

Fig. 4. (A) Light micrograph of a shallow, full-thickness defect 8 weeks after treatment of the bony portion with a chondrogenic matrix lacking suramin and of the cartilaginous compartment with the same chondrogenic matrix containing free suramin. Despite the presence of free suramin, the osteogenic repair response (O) emanating from the subchondral bone bed (S) still encroached on the cartilaginous defect space, although to a lesser degree than when no antiangiogenic factor was included in the chondrogenic matrix (as shown in Fig. 3). The remainder of the cartilaginous compartment is occupied by a mixture of primitive mesenchymal and cartilage-like repair tissue (R), which is readily distinguishable from native hyaline cartilage (N). An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=150 µm. (B) Light micrograph of a shallow, full-thickness defect 8 weeks after receiving the treatment described in (A). In this example, osseous tissue (O) occupies a larger proportion of the cartilaginous compartment than is the case in (A). The remainder of this compartment contains a mixture of primitive mesenchymal and cartilage-like repair tissue (R), similar in composition to that present in (A). S, subchondral bone; N, native hyaline cartilage. An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=200 µm.

The current study shows that the functional barrier principle can be implemented with good effect in the growth-factor-based treatment of full-thickness defects. Its advantages over the structural barrier principle are that it triggers no inflammatory response at the interface between cartilaginous and bony compartments and that it permits better integration between repair tissue types at this junction. As a result, repair tissue would be expected to have greater mechanical competence. However, this concept has been tested only in a small, full-thickness defect model.

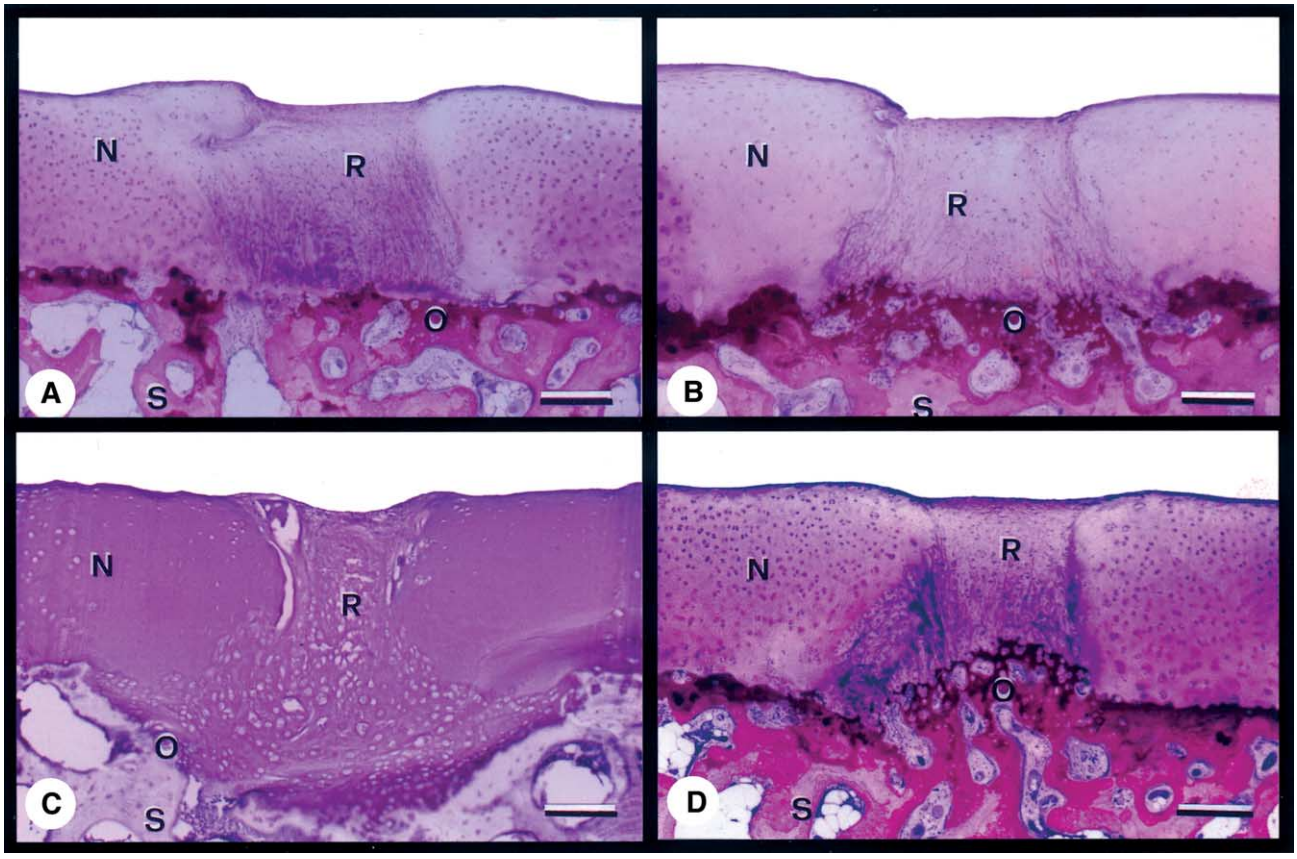

Fig. 5. (A) Light micrograph of a shallow, full-thickness defect 8 weeks after treatment of the bony portion with a chondrogenic matrix lacking suramin and of the cartilaginous compartment with the same chondrogenic matrix containing free and liposome-encapsulated suramin. The osteogenic repair response (O) stemming from the subchondral bone bed (S) is confined to the bony portion of the defect. The cartilaginous compartment is filled exclusively with a mixture of primitive mesenchymal and cartilage-like repair tissue (R). This finding indicates that when present in free and liposome-encapsulated forms, suramin completely inhibits vascular invasion of, and bone formation in, the cartilaginous compartment. In this example, the regenerated interface between bony and cartilaginous compartments is flush with the natural junction. N, native hyaline cartilage. An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=250 µm. (B) Light micrograph of a shallow, full-thickness defect 8 weeks after receiving the treatment described in (A). As in (A), the osteogenic repair response (O) emanating from the subchondral bone tissue (S) is restricted to the bony portion of the defect. The cartilaginous compartment is filled entirely with a mixture of primitive mesenchymal and cartilage-like repair tissue (R), which is similar in composition to that present in (A). The regenerated cartilage–bone interface is flush with the natural boundary. N, native hyaline cartilage. An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=200 µm. (C) Light micrograph of a shallow, full-thickness defect 8 weeks after receiving the treatment described in (A). In this instance, the bony defect space was slightly underfilled with the chondrogenic matrix, such that the regenerated cartilage–bone interface is depressed relative to the natural border. As in (A) and (B), the osteogenic repair response (O) is confined to the bony compartment. The cartilaginous compartment is filled exclusively with a mixture of primitive mesenchymal and cartilage-like repair tissue (R), with the latter predominating. N, native hyaline cartilage; S, subchondral bone; An 18-µm-thick cryosection stained with toluidine blue O. Bar=150 µm. (D) Light micrograph of a shallow, full-thickness defect 8 weeks after receiving the treatment described in (A). In this example, the bony defect space was slightly overfilled with the chondrogenic matrix, such that the regenerated cartilage–bone interface is elevated relative to the natural border. As in (A–C), the osteogenic repair response (O) is restricted to the bony portion of the defect. The cartilaginous compartment is filled entirely with a mixture of primitive mesenchymal and cartilage-like repair tissue (R). N, native hyaline cartilage; S, subchondral bone. An 80-µm-thick section, surface-stained with McNeil's tetrachrome, toluidine blue O, and basic fuchsin. Bar=250 µm.

Additional studies are needed to adapt this concept to larger lesions.

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