Incorporation of cortical bone allografts and autografts in rats

Expression patterns of mRNAs for the TGF-ßs

Petri Virolainen[1,2], Kati Elima[2], Marjo Metsäranta[2], Hannu T Aro[1] and Eero Vuorio[2]

Healing of bone grafts is dependent on the rate of new bone formation. To understand better the regulation of new bone formation in the graft we have studied local production of TGF-ß1, 2 and 3, and of the small proteoglycans by determining their mRNA levels in a rat bone graft model. These mRNA levels were compared to the healing rates of autografts and allografts, as determined by histology, UV-microscopic evaluation of tetracycline-labeled new bone formation, microradiography and mechanical testing at 1, 2, 4 and 8 weeks of healing. Analyses showed that, analogous to slower bone formation in allografts, the induction of TGF-ß1 gene expression was slower than in allografts, when compared with autografts. A similar delay was seen in decorin gene expression. The results agree with the suggested role of TGF-ß1 in induction of type I collagen and osteonectin production. Our findings thus support the view that locally produced TGF-ß1 plays a role in normal graft incorporation, while local production of TGF-ß3, and particularly TGF-ß2, may be less important in this respect.

Departments of [1]Surgery, and [2]Medical Biochemistry and Molecular Biology, University of Turku, FI-20520 Turku, Finland. Correspondence: Dr. Petri Virolainen, Department of Surgery, Kiinamyllynkatu 8–10, University of Turku, FI-20520 Turku, Finland. Tel +358 2-261 1611. Fax -333 7229
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Incorporation of cortical bone grafts proceeds slowly. Although bony union across the graft-host interface is achieved within a few weeks, large grafts never become fully populated by host osteoblasts (Burchardt 1983). The structural properties of such grafts are therefore largely dependent on the long half-lives of their constituent matrix components. Since host osteoblasts are scarce, microtrauma of grafted bone may not heal adequately, but result in gradual weakening of grafts. One approach for improving graft incorporation is administration of factors capable of stimulating the invasion and remodeling of the graft by host osteoclasts and osteoblasts. A multitude of hormones and growth factors have been characterized which affect bone formation and resorption (Reddi 1992, Rosen and Thies 1992). Many of these factors are believed to act in concert, but the hierarchy of their action is poorly understood.

The transforming growth factor-ßs (TGF-ßs) are a large family of multifunctional growth factors well known for their capacity to stimulate extracellular matrix production in bone and other connective tissues (Centrella et al. 1994). Currently, three TGF-ß isoforms, TGF-ß1, TGF-ß2 and TGF-ß3, are known in mammals exhibiting different temporal and spatial expression patterns, with considerable overlap in essentially all human and murine tissues (Miller et al. 1989a, b, Gatherer et al. 1990, Schmid et al. 1991). Differential expression of the three TGF-ß mRNAs in bone, periosteum and cartilage suggests that the individual TGF-ßs have specific functions in these tissues (Centrella et al. 1994). Several experiments have shown that injections of TGF-ßs and other growth factors belonging to the TGF-ß superfamily (e.g., the bone morphogenic proteins, BMPs) into bone defects or fracture sites have a stimulatory effect on bone formation (Reddi 1992, Lind et al. 1993). These observations are in contrast with data from studies on TGF-ß1 knock-out mice; transgenic mice homozygous for TGF-ß1 null allele exhibit normal skeletal development and die at the age of 3-4 weeks, with a wasting syndrome due to an excessive autoimmune-like inflammatory reaction (Shull et al. 1992, Kulkarni et al. 1993). This demonstrates considerable redundancy of the TGF-ß family in bone and cartilage and most other tissues. Nevertheless, the fact that the highest concentrations of TGF-ßs in the body are found in bone and in platelets provides additional indirect evidence for the biological importance of these factors for bone healing and remodeling.

We have previously used a molecular biologic approach to study bone graft incorporation, employing a

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rat model, and demonstrated that the differential healing rates of allografts and autografts are reflected in the gene expression patterns (mRNA levels) of type I collagen fibrillar collagens and osteonectin (Virolainen et al. 1993, 1995). The mRNA levels of these key components of bone matrix thus predict retarded union strength of the graft-host interface. Little is known of endogenous TGF-ß production during normal and delayed bone graft incorporation and since the three small proteoglycans, decorin, biglycan and fibromodulin, are capable of binding TGF-ßs their expression patterns were also analyzed. We feel more information is needed on the relationship of TGF-ß and small proteoglycan production and histologic and mechanical healing rates of bone grafts, before we can successfully interfere with the process.

Animals and methods

This study is based on the analysis of 252 cortical bone grafts in 63 Sprague-Dawley rats weighing 224–533 grams at the end of the experiment. Half of the grafts were autografted and the other half allografted. Grafts in 43 rats were used for descriptive histologic, microradiographic and mechanical analyses. 10 allografts and autografts from time-points of 1, 2 and 4 weeks and 12 grafts from a time-point of 8 weeks were used for mechanical analysis. Bone samples from 4 non-operated animals were used as controls for normal bone. 10 other allografts and autografts were divided into those used for histological analysis, 5 allografts and autografts were used for UV-light microscopy and microradiography and 5 samples (allografts and autografts) were sectioned and stained with toluidine blue. Grafts in another series of 20 rats were used to isolate total RNA. 10 allografts and autografts from each time-point were pooled together.

Bone healing model

Surgery was performed under general anesthesia, using a combination of metotidim and ketamine. The bone healing model consisted of two round unicortical defects 3 mm in diameter in the proximal tibia (Figure 1). The defects were autografted or allografted with 3 mm round blocks of cortical bone. Each animal received 4 grafts: 2 cortical autogenous grafts to the left tibia and 2 frozen cortical unmatched allografts for the right tibia. The grafts were removed, using a round dental drill under saline cooling. The autografts were taken from the right tibia and transplanted immediately into the left tibia. Similarly, the right tibia received 2 allografts which had been harvested from antigen-unmatched animals and stored at

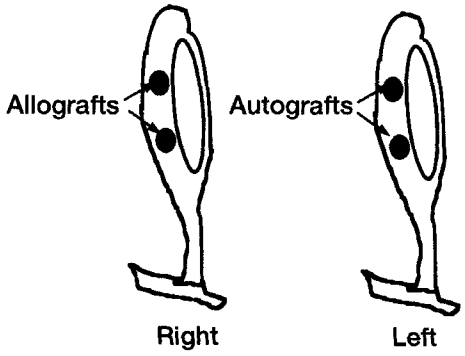

Figure 1. Schematic presentation of the bone defect. The defect was 3 mm in diameter. On the right it was filled with bone allografts of the same size and on the left with bone autografts.

–20 °C for 2 weeks before implantation. No external or internal fixation was needed. Weight bearing was not restricted after surgery. The animals were killed at 1, 2, 4 and 8 weeks after the operation. The tibias were harvested and the grafts were randomly selected for comparative histologic analysis, mechanical testing and microradiography.

Histologic analysis

The undecalcified samples were fixed in 70% alcohol, dehydrated and defatted in increasing concentrations of alcohol and embedded in methacrylate. Sections of 20 and 80 µm were cut with a microtome. The 20 µm sections were stained with toluidine blue for normal light microscopy. The 80 µm slides were examined with UV-light microscopy to evaluate tetracycline-labeled new bone formation. For this, the test animals received an intramuscular injection of oxytetracycline 25 mg/kg at the time of surgery and once a week thereafter. The same 80 µm sections were also analyzed with contact microradiography.

Mechanical testing

Tibial specimens were stored frozen at –23 °C for a maximum of 2 weeks before mechanical testing. For testing, the specimens were thawed at room temperature, and kept moist with saline solution through all stages of handling. Each specimen was rigidly mounted to the testing surface with bone cement. Standardized indentation measurement of the grafts was then performed, using a mechanical testing device (Avalon Technologies, Rochester, MN, USA). The indentation test was used to determine the material properties of the grafts at various stages in the healing process (1, 2, 4 and 8 weeks).

The area of interest in the center of the graft was loaded under axial compression, using a cylindrical indentor, 1.6 mm in diameter. A constant slow deformation rate of 1 mm/min was used. The loading was

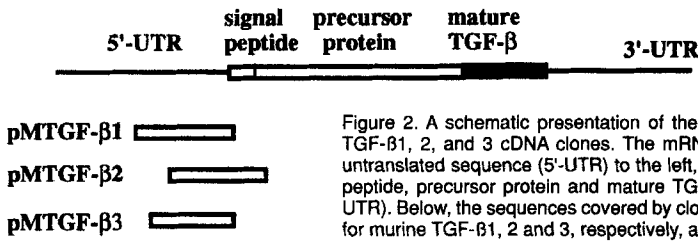

Figure 2. A schematic presentation of the consensus TGF-ß mRNA and the murine TGF-ß1, 2, and 3 cDNA clones. The mRNA for TGF-ß is shown at the top with 5'-untranslated sequence (5'-UTR) to the left, followed by the coding sequence for signal peptide, precursor protein and mature TGF-ß, and the 3'-untranslated sequence (3'-UTR). Below, the sequences covered by clones pMTGF-ß1, pMTGF-ß2 and pMTGF-ß3 for murine TGF-ß1, 2 and 3, respectively, are shown in relationship to the mRNA.

continued until either a maximum fixed depth of 0.5 mm was reached or an obvious failure (yield point) was observed. Structural deformation of the cortical bone graft, calculated from the impression of the indentor during loading, was continuously recorded, with the load applied. In analyzing the load-deformation curves, the modified Brinell hardness value was calculated, using the following formula:

modified Brinell hardness value = load / ((π × diameter of indentor / 2) × depth of impression)

Control cortical bone specimens from intact rat tibias (n 12) were sectioned, frozen, stored and measured similarly.

Construction of hybridization probes for murine TGF-ßs

In order to characterize the expression of TGF-ß1, 2 and 3 genes in rodent samples short cDNA probes for mouse TGF-ßs were constructed. For this purpose, total RNA and DNA were extracted from limb cartilages of newborn mice, using the guanidine isothiocynate method (Sambrook et al. 1989). Single-stranded cDNA was synthesized for the RNA template, using Malone Murine Leukemia virus (MMLV) reverse transcriptase, random hexamers and oligo(dT) for primers, under conditions suggested by the supplier (Gene Amp® RNA PCR kit, Perkin-Elmer, Norwalk, CT, USA). Both this cDNA and genomic DNA were subsequently used for PCR amplification of TGF-ß1, 2 and 3 sequences, using two 17-mer oligonucleotide primers for each TGF-ß. Due to the high degree of similarity between the coding regions of the different TGF-ßs (Derynck et al. 1986, Miller et al. 1989a, b), the oligonucleotides used for the amplifications were chosen from the 5'-untranslated region of the mRNAs, extending into the sequence coding for the precursor peptide domain, where the three genes are divergent (Figure 2). As the homology between the rat and mouse sequences in this region turned out to be 95%, we chose to amplify only the murine sequences, since these would serve as specific hybridization probes for both species.

The 402 base pair (bp) TGF-ß1 cDNA fragment obtained was cloned by blunt-end ligation into the EcoRV site of Bluescript SK (Stratagene, La Jolla, CA, USA) and the corresponding 387 bp TGF-ß2 and 323 bp TGF-ß3 cDNAs in pGEM®-T (Promega, Madison, WI, USA). Both vectors allow easy preparation of single- and double-stranded DNA and of sense and antisense RNA. All inserts were identified by sequencing, employing the Sequenase® reagent kit (United States Biochemical, Cleveland, OH, USA), which confirmed their identity with the published mouse TGF-ß cDNA sequences (Derynck et al. 1986, Miller et al. 1989a, b).

Gene expression studies

For the extraction of total RNA, 8–10 samples were pooled for each time-point to obtain sufficient material for Northern analyses. Bone tissue was ground into fine powder under liquid nitrogen in a mortar and transferred into 4 M guanidinium isothiocyanate solution for isolation of total RNA (Sambrook et al. 1989). For Northern blots, the total RNAs were denatured with glyoxal and dimethylsulfoxide, electrophoresed on 0.75% agarose gels, and transferred by blotting onto Pall Biodyne A transfer membranes (Pall, Portsmouth, England). The membranes were prehybridized and hybridized with ^{32}P-labeled probes. After hybridization, the filters were washed and the bound probe detected by autoradiography and quantified by laser densitometry. Equal loading of the gels was ascertained by measurement of the rRNAs fractionated on duplicate gels, stained with ethidium bromide. Inserts were purified from each plasmid, after appropriate restriction digests, and labeled with ^{32}P-dCTP to specific activities of approximately 10^8 cpm/µg, using the random priming method. In addition to the cDNA probes for TGF-bs described above, the following probes were used: pMDcn-1 for mouse decorin mRNA, pMBgn-1 for mouse biglycan mRNA, pMFmn-1 for mouse fibromodulin mRNA.

Statistics

The statistical comparison of the indentation hardness between allografts and autografts was performed with ANOVA.

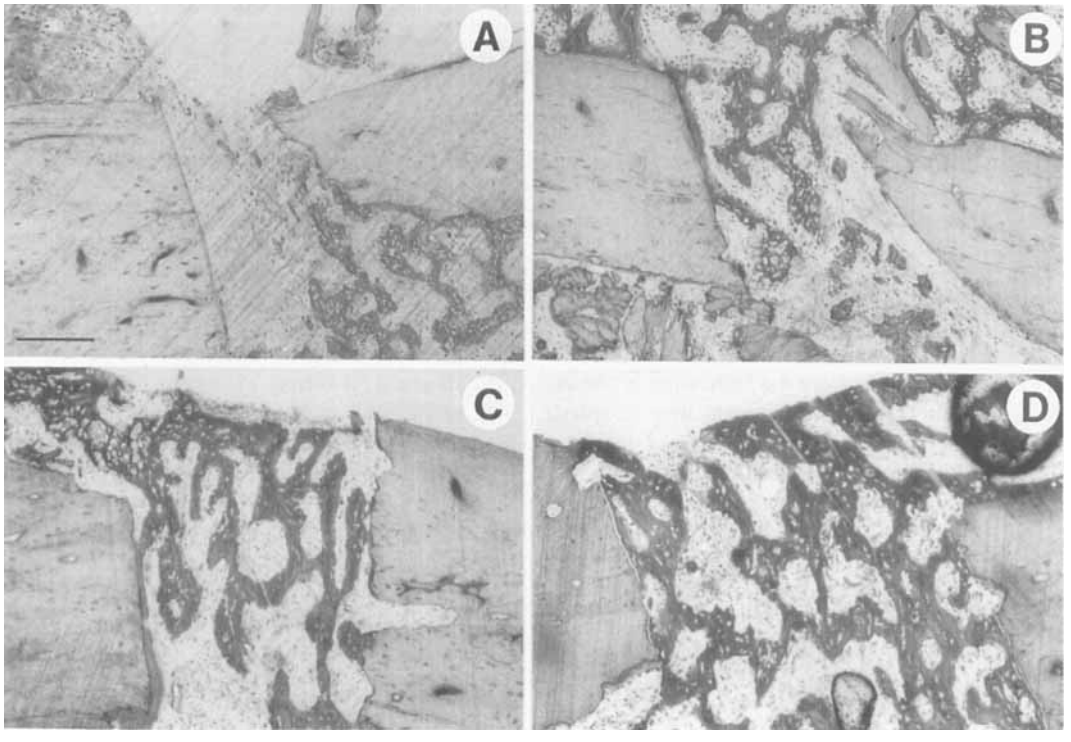

Figure 3. Histology of the graft-host interface at 1 and 2 weeks of allograft and autograft incorporation. In each panel the host bone is to the left, the grafted bone to the right, and the marrow cavity to the bottom. Panel A, allograft at 1 week of healing; panel B, autograft at 1 week of healing; panel C, allograft at 2 weeks of healing; and panel D, autograft at 2 weeks of healing. Toluidine blue staining. The bar in panel A corresponds to 100 μm.

Results

Histology and microradiography

Descriptive histologic analyses confirmed that the onset of the healing process occurred consistently earlier in autografts than in allografts. Creeping substitution by vascular canals and resorption cavities was the commonest healing mechanism in autografts. This was also the case with some allografts. However, all three healing mechanisms described earlier (Burchardt 1983, Friedlaender 1991, Virolainen et al. 1993) were seen in allograft incorporation. Some allografts were rapidly and almost totally revascularized and resorbed, while in most grafts the resorption was only partial and the remaining nonvascularized allograft was surrounded by new bone. In all grafts, the invasion of the graft-host interface by mesenchymal cells from the host was seen as an early healing step. At one week, the mesenchymal reaction was stronger in autografts than in allografts, indicating an earlier onset of the process in the former. Analogously, formation of a bony bridge between graft and host bone occurred earlier in autografts than in allografts (Figure 3).

Tetracycline labeling and UV-microscopy revealed that the replacement of the original graft by new bone was less complete in allografts than in autografts. During the first 2 weeks, both types of grafts were replaced by new bone only superficially. At 8 weeks, new bone formation both in allografts and in autografts was still most extensive in superficial areas; however, in autografts abundant new bone formation was also observed in central areas of the graft (Figure 4).

Microradiographic analysis confirmed that solid bony union was achieved at the graft-host interface both in autografts and in allografts. Graft porosity, indicating vascular invasion, appeared greater in autografts, while resorption at the graft surface was more extensive in allografts (Figure 5). By 8 weeks, a complete bony union was seen at each graft-host interface.

Mechanical properties

No statistically significant differences were observed in the indentation stiffness between allografts and autografts at any of the time-points (Figure 6). Although the hardness values differed somewhat between the different time-points during the 8-week study period, none of the changes reached statistical significance.

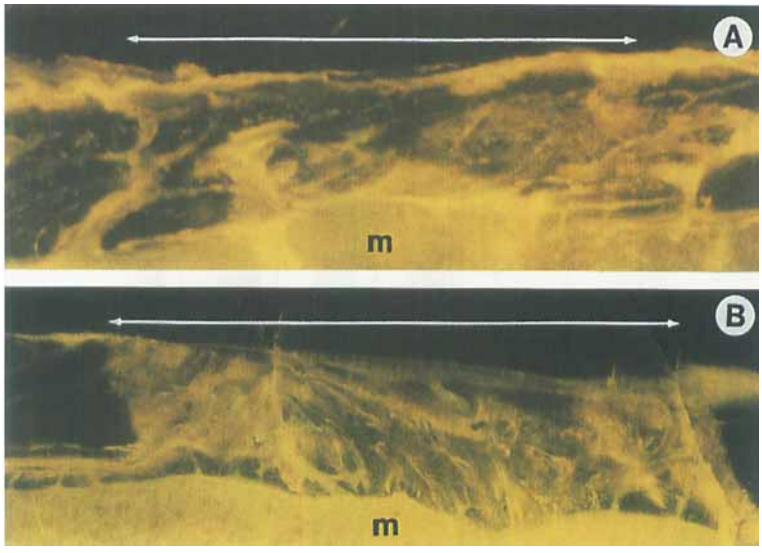

Figure 4. UV microscopic appearance of the allograft (A) and autograft (B) at 8 weeks of graft incorporation. The entire graft (arrow), both graft-host interfaces and the adjoining host bone, is shown with the marrow cavity (m) to the bottom. Within the bone, the fluorescence denotes new bone formation. The diameter of the graft marked with the arrow corresponds to 3 mml.

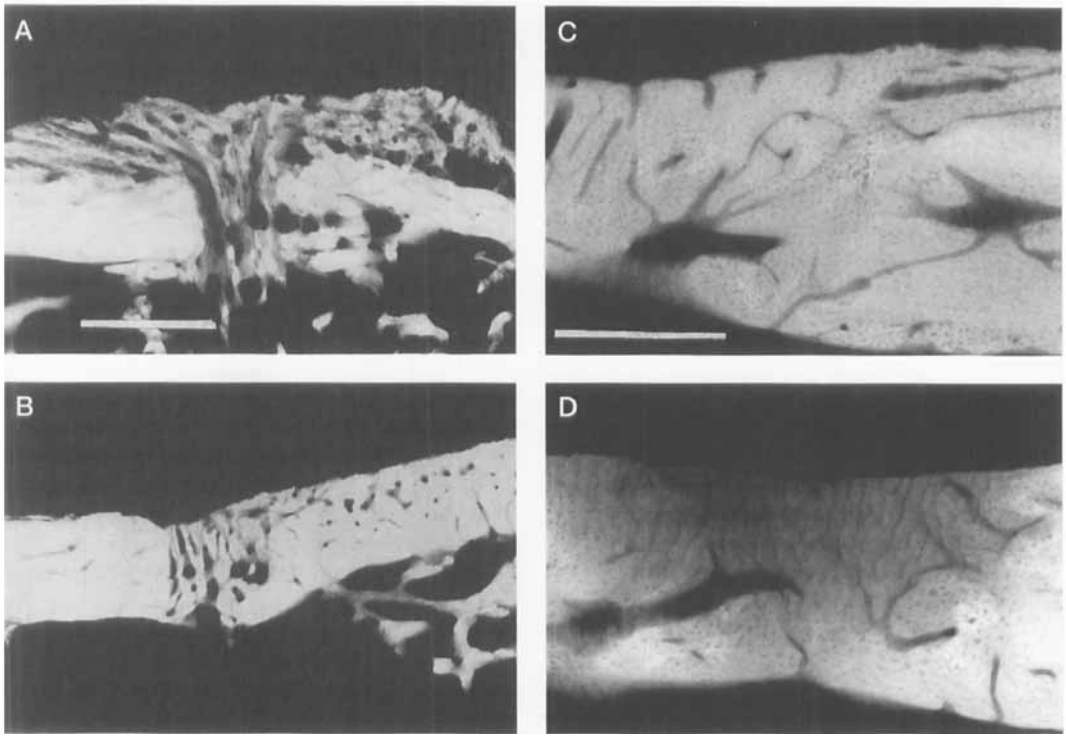

Figure 5. Microradiography of the graft-host interfase at 4 and 8 weeks of graft incorporation. A, allograft at 4 weeks of healing; B, autograft at 4 weeks of healing; C, allograft at 8 weeks of healing; and D, autograft at 8 weeks of healing. The interface is shown in the middle, the host bone to the left, the graft to the right, and bone marrow to the bottom. The bar in figure A (for A and B), corresponds to 1 mm, and in figure C (for C and D) to 250 µm.

Expression of TGF-ß and small proteoglycan genes during graft healing

In autografts, expression of the TGF-ß1 gene reached its maximal level during the first week, while in allografts, the maximal level was lower and was reached at 2 weeks of healing. After these early phases of graft-host incorporation, the TGF-ß1 mRNA levels declined and the difference between allografts and autografts diminished (Figure 7A). The levels of TGF-ß3 mRNA were not so high as those of TGF-ß1.

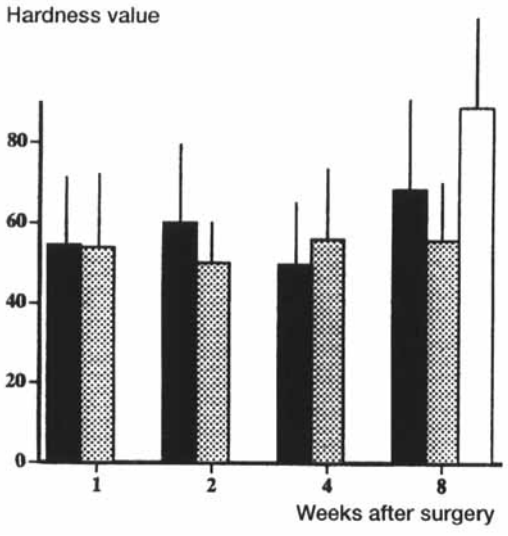

Figure 6. The mechanical properties of cortical bone allografts and autografts, expressed as N/mm^2 (modified Brinell's hardness value). Black bars, allograft; dotted bars, autografts; open bar, intact bone. Each bar represents the mean ± SD.

Although their expression pattern was similar to TGF-ß1 mRNAs, the levels were too low for reliable quantitation. TGF-ß2 mRNA levels were not detectable at any of the time-points studied.

Initially, decorin mRNA levels were higher in autografts than in allografts (Figure 7B). In allografts, decorin mRNA levels remained constant during the whole 8-week study period, while in autografts, more variation was observed. Biglycan mRNA levels were similar in allografts and autografts (Figure 7C). In both groups, the levels declined during the healing process; by 8 weeks, the mRNA levels were about 50% of those at one week of healing. Fibromodulin mRNA was undetectable during the whole study period in both groups.

Discussion

The animal model we used enabled us to study normal and delayed graft-host incorporation by comparing autografts to allografts, using molecular biologic techniques with different histologic, radiographic and mechanical analyses. Since bone graft incorporation has been studied in great detail, both histologically and mechanically (Burchardt 1983, Friedlaender 1991), we concentrated on molecular biology and used descriptive histology only to document a normal healing pattern. The usefulness of the model is also demonstrated by the low complication rate. Although no graft fixation was used, graft displacement occurred in only 2 grafts.

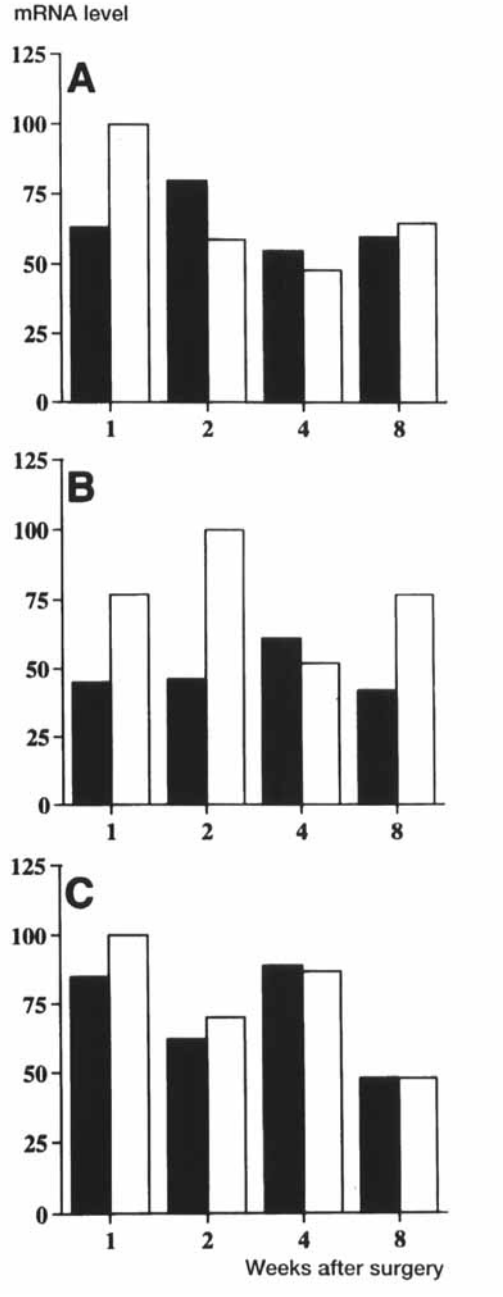

Figure 7. Northern analysis of mRNA levels for TGF-ß1, decorin and biglycan during allograft and autograft incorporation. Total RNAs (10 µg each) isolated from pooled samples at time-points indicated below were fractionated on 0.8% agarose gels, transferred onto Pall Biodyne membranes and hybridized with cDNA probes for TGF-ß1 (panel A), decorin (B) and biglycan (C). Hybridization was detected by autoradiography and quantified by laser densitometry. The mRNA levels are given in relative units calculated per rRNA, as determined by ethidium bromide staining of parallel gels. Black columns, allograft; open columns, autografts.

Histological examination showed three different healing mechanisms of bone allografts, as described earlier (Burchardt 1983, Friedlaender 1991, Virolainen et al. 1993). These mechanisms have been said to be correlated with the different immunogenic properties of the unmatched allograft (Friedlaender 1991). Enneking and Mindell (1991) showed, in a human material, that only 20% of large bone allografts is replaced by new bone during the first 5 years. Although the size of cortical grafts in our study was relatively small, the results agree with these findings. At 8 weeks, UV-microscopy showed that both allografts and autografts were mixtures of necrotic old graft bone and new viable bone. The replacement was more complete in autografts. This may be the primary reason for the problems related to late mechanical failures of structural allografts. In autografts, the amount of viable bone, capable of repairing microfractures is greater than in allografts.

Our previous studies showed that the strength of the graft-host union increased more slowly in allografts than in autografts. The final outcome, however, was the same (Virolainen et al. 1993). Using the modified Brinell hardness value, the current study extended the mechanical analyses into the material properties of the graft itself. This method has several limitations, but is widely used in mechanical studies of bone quality (Markel et al. 1990). The main disadvantage of the method is the high variation of bone stiffness properties, even within small regions of interest. This was also observed in the current study. 1 week after grafting, the hardness of the grafts tended to be less than that of intact frozen bone graft. Since freezing of bone grafts does not have bad effects on the mechanical properties of bone graft (Pelker et al. 1984), the loss of stiffness was obviously due to increased internal porosity. Enneking et al. (1975) have shown in an experimental model (in dogs) that cortical grafts weakened by approximately 40% from normal up to 6 months after grafting because of increased internal porosity. Our results agree with those findings, although the reduction in hardness values was not so striking in rats. However, necrotic allografted bone is more prone to fatigue damage than viable bone. Such liability to fatigue damage is unlikely to be shown by direct measurement of hardness. Tests of repetitive loading could have given us different results.

The slower incorporation rate of allografts, reflected in lower initial mRNA levels of major components of bone matrix, type I collagen and osteonectin (Virolainen et al. 1993, 1995), was also seen as delayed induction of TGF-ß1 gene expression. A similar delay was seen in decorin gene expression. These results accord with the suggested role of TGF-ß1 in induction of type I collagen, small proteoglycan and osteonectin production (Robey et al. 1987, Hardingham and Fosang 1992). No differences were detected between the autograft and allograft groupsin the levels of TGF-ß3 mRNA. TGF-ß2 and fibromodulin mRNAs remained undetectably low during the entire observation period. Schmid et al. (1991) found that in embryogenesis all three TGF-ß isoforms are expressed during endochondral bone development, but only TGF-ß1 and TGF-ß3 are involved in intramembranous bone formation. They suggested that the absence of TGF-ß2 during certain stages of development may result in ossification, without a prior cartilage matrix. This may explain why TGF-ß2 was not detectable in these series of studies, as graft healing proceeds by straight intramembranous bone formation. Of the small proteoglycans, fibromodulin is specific to cartilage; analogously, no fibromodulin mRNA was detected during graft incorporation. This agrees with our earlier observations of the absence of the cartilage-specific type II collagen mRNA at the graft-host interface (Virolainen et al. 1993, 1995).

During normal fracture healing in rats, the expression of TGF-ß has two peaks at 5 and 15 days after fracture (Joyce et al. 1990). The first peak was thought to correspond to osteoblast activity associated with intramembranous (appositional) bone formation, and the expression on day 15 to osteoblast activity during endochondral ossification. Since no endochondral ossification occurs during graft incorporation, our observation of the highest level of TGF-ß1 expression during the first week is consistent with these observations and interpretations. The present data thus support the view that locally produced TGF-ß1 plays a role in normal graft incorporation, while local production of TGF-ß2 and TGF-ß3 appears to be less important for the formation of bony union. However, as both bone matrix and platelets are rich sources of TGF-ßs (Seyedin et al. 1985), liberation of active growth factors from these sources could be important for initiation of the healing response. Both TGF-ß1 and TGF-ß2 have also been isolated as cartilage-inducing factors A and B, based on their ability to induce cartilage formation (Seyedin et al. 1985). Chondrogenesis was never observed in our graft healing model. To us this suggests that the microenvironment and the presence of other growth factors at the repair site, together with TGF-ßs, regulate the osteogenic and chondrogenic differentiation programs of the mesenchymal cells during skeletal repair.

The three small proteoglycans, decorin, biglycan and fibromodulin, appear to play two roles in the assembly of skeletal matrices. All are involved in cartilage and/or bone formation, through their capacity to

associate with fibrils of type I and type II collagen (Scott 1988). Decorin and biglycan are found in bone, while all three appear to be present in cartilage (Bianco et al. 1990, Fedarko et al. 1990) The small proteoglycans can also bind growth factors of the TGF-ß family and are therefore involved in the modulation of many cellular functions (Kähäri et al. 1991). This study showed that the expression pattern of decorin gene was associated with that of TGF-ß1 gene. The delay in TGF-ß1 gene expression was similar to the delay in decorin expression and these two phenomena are probably linked to each other. This study clearly showed that different TGF-ßs and proteoglycans have different expression patterns during bone graft healing. Several experimental designs have shown that injections of growth factors belonging to the TGF-ß superfamily (TGF-ßs and BMPs) into bone defects or fracture sites have a stimulatory effect on bone formation (Joyce et al. 1990, Lind et al. 1993). However, their efficiency has not yet been tested with bone grafts. Since administration of TGF-ßs may also result in severe fibrosis of heart, kidneys, lungs and bone marrow and in excessive periosteal bone formation (Border and Noble 1994), their dosage also needs to be carefully adjusted before medical use. Our graft model presented here should be useful for such testing.

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