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Expression of tissue inhibitor of metalloproteinase during early stages of bone graft healing

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1. ABSTRACT

The aim of this study was to investigate the temporal expression of TIMP-1 within endochondral and intramembranous bone grafts during the early stages of healing in thirty six adult New Zealand white rabbits. Total RNA was isolated from bone grafts extracted on days 0-11 and day 14 post-grafting, for RT-PCR analysis. *In situ* hybridization was carried out on days 1-9 and day 14. Results showed TIMP-1 expression coincides with osteogenesis, which indicates a role for TIMP in preserving the newly formed bone during the initial stages of graft healing. Bone grafts play an important role in influencing the healing process mediated by the host tissues. The temporal expression of TIMP-1 differs in endochondral and intramembranous bone grafts. The earlier expression of TIMP-1 by endochondral bone grafts, could be the reason for the delayed vascularization while the expression of TIMP-1 by the intramembranous bone grafts, at a later stage could allow for earlier vascularization.

2. INTRODUCTION

Bone graft healing is a complex process involving the degradation of surface osteoid and mineralized matrix by matrix metalloproteinases (MMPs) (1). MMPs are a family of zinc dependent proteinases capable of degrading connective tissue, and are involved in bone matrix degradation during osteogenesis and remodelling (2). MMPs are secreted from cells in the form of pro-MMPs and activated by disruption of the Zn2+–cysteine bond which blocks the active site (1,3). There are several groups of MMPs; the collagenases (MMPs 1, 8, 13, 18), which degrade interstitial collagens (types I–III); the gelatinases (MMPs 2, 9), which degrade collagen type IV and gelatin; the stromelysins (MMPs 3, 10, 11) which are more general proteinases and degrade proteoglycan, fibronectin, laminin, gelatin, casein and some collagens; the membrane type MMPs (MMPs 14, 15, 16, 17, 24, 25); the minimal-domain MMPs (MMPs 7, 26) and others (MMPs 12, 19, 20, 21, 22, 23, 27, 28) There is considerable redundancy and overlap between them with respect to their function. (1,3,4,5).

The activity of MMPs is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) (1,6,7). TIMPs inactivate MMPs by forming non-covalent bimolecular complexes and prevent pro-MMP activation (6). There are four members of the TIMP family (TIMPs 1, 2, 3, 4), of which TIMP-1 has been the most extensively studied (7). TIMPs and MMPs have been implicated to play important roles in bone formation and remodelling (4,7) and have been evaluated in the embryonic rabbit mandible, human osteophytic bone, neonatal rib and heterotopic bone (1,7).

TIMP-1, the predominant form of the TIMPs, has been shown to inhibit most MMPs including MMP-2, MMP-3, and MMP-13 (8,9) In addition, TIMP-1 can form a specific complex with latent MMP-2 that leads to stabilization of the enzyme activation mechanism. Several previous reports have shown that TIMP-1 and TIMP-2 can modulate osteoclastic bone resorption *ex vivo*. The more convincing reports showed that recombinant TIMP-1 and TIMP-2 can inhibit PTH- or vitamin D3–stimulated bone resorption in organ cultures (10). Another report presented TIMP-1 and TIMP-2 as acting either as stimulators or inhibitors of bone resorbing activity on purified mature osteoclasts *in vitro*, depending on their concentration; only higher concentrations produced osteoclast activity inhibition (11,12). These data highlight the crucial influence of the level of expression of the TIMPs in the modulation of bone graft healing. In addition, studies in humans also have shown that the level of TIMP-1 is important in ensuring properly organized bone formation. Indeed, in pathological samples of heterotopic and osteophytic bones, the lack of TIMP-1 expression can partly explain the poorly organized bone (7).

The temporal expression of MMPs and TIMPs in bony defect following bone grafting with intramembranous and ectochondral bone remains unknown. Therefore, the objectives of this study are:

to determine the temporal pattern of expression of TIMP in endochondral bone graft and to determine the temporal pattern of expression of TIMP in intramembranous bone graft hoping to provide an understanding on the effect of TIMP on the integration of bone grafts.

3. MATERIALS AND METHODS

The rabbits were divided into twelve groups, each consisted of three rabbits, representing days 1 to 11 and day 14 post-operatively on sacrificing. Seventy two 10×5mm full-thickness bone defects were created in the parietal bones of 36 New Zealand White rabbits from an inbred colony. The rabbits were 5 months old (adult stage) and weighed 3.5-4.0 kg. The handling of the animals and the experimental protocol were approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong. Each rabbit was grafted with IM bone in one defect and with EC bone in the other. Rabbits were killed on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 14 post-grafting and the bone graft alone was harvested for mRNA analysis. Grafts were harvested from at least three different rabbits for each time point and total RNA was extracted and processed separately.

3.1. Surgical Procedures

The details of the operation and the postoperative care of the animals were previously described (13). In short, the animals were premedicated 1 hour before surgery with oxytetracycline hydrochloride (200mg/ml, 30mg/kg body weight, Tetroxyla, Bimeda, Dublin, Ireland) and buprenorphine hydrochloride (0.3ml/kg body weight, Hypnorm, Janssen Pharmaceutical, Beerse, Belgium), supplemented with diazepam (5mg/ml, 1mg/kg body weight, Valium 10, Roche). In order to maintain the level of neuroleptanalgesia, increments of Hypnorm (0.1ml/kg) were given at 30-min intervals during the operation. The surgical procedure consisted of the creation of one or two 10×5mm full-thickness (approximately 2mm) cranial defects, devoid of periosteum, using templates, in the parietal bones. A piece of cranial IM bone was grafted to one defect and EC bone of an identical size harvested from the diaphyseal tibial shaft to the other defect. Holes (approximately 1 mm) were drilled at opposite ends of the bone grafts and likewise in the parietal bone to allow for fixation of the bone grafts with stainless steel wire (0.3 mm).

3.2. Postoperative care

All wounds were closed with interrupted 3/0 black silk sutures. No attempt was made to approximate the periosteum to prevent the barrier effect. Postoperatively, the rabbits were given oxytetracycline hydrochloride daily for up to 10 days and buprenorphine hydrochloride for up to 2 weeks.

At the predetermined date after surgery, the animals were killed with sodium pentobarbitone. Immediately upon death, defects and surrounding tissue were removed for histological and molecular analysis.

3.3. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of TIMP-1

RT-PCR, the bone graft alone was harvested on days 1 to 11 and day 14 post-grafting in order to examine the temporal expression of TIMP-1 mRNA.

Total RNA for reverse transcriptase polymerase chain reaction (RT-PCR) analysis of TIMP-1 was extracted from bone using a modified version of Chomczynski and Sacchi's (14) single step AGPC (acid guanidinium thiocyanate-phenol-chloroform) protocol.

First-strand cDNA was synthesised from total RNA using a Ready-To-Go™ T-Primed First-Strand Kit (Pharmacia Biotech Inc.). RT-PCR was carried out in a 20 µl reaction solution containing 1 x PCR buffer (20mM Tris.HCl pH 8.4, 50mM KC1; Gibco BRL, Life Technologies), 4.5mM $MgCl_2$ (Gibco BRL, Life Technologies), 0.2mM of each dNTP (Pharmacia Biotech), 1 ng of each primer (Table 1; Gibco BRL, Life Technologies), 2.5 units Taq DNA polymerase (Gibco BRL, Life Technologies) and 1ml template cDNA.

Table 1. Oligonucleotide primers used for PCR

Target	Sense primer (5' to 3')	Anti-sense primer (5' to 3')	Product Size
GAPDH (Control)	TAT GAC GAC ATC AAG AAG GTG G (831-852bp)	CTT GGA GGC CAT GTG GAC (1070-1053bp)	239bp
TIMP-1	C ACC TGT GTC CCA CCT CAC (87-105bp)	TGT CAC TCT CCA GAT GGC AG (511-492bp)	425bp

Table 2. Oligonucleotide primers used for amplifying DNA fragments

Primer	Sequence (5' to 3')
SP6(Forward)	TAT TTA GGT GAC ACT ATA G
T7(Reverse)	GTA ATA CGA CTC ACT ATA GGG C
TIMP-1(Forward)	C ACC TGT GTC CCA CCT CAC
TIMP-1(Reverse)	TGT CAC TCT CCA GAT GGC AG

Amplification was carried out using a Thermal Sequencer with heated lid (MJ Research, Inc. Massachusetts, USA). Template cDNA was completely denatured by a 3-minute incubation at 94°C and was followed by 30 amplification cycles. Conditions for amplification consisted of a 30 second denaturation at 94°C, a 2 minute primer annealing step at 60°C and a 2 minute extension step at 72°C. After the last cycle the samples were incubated at 72°C for 7 minutes in order to fill in the protruding ends of the newly synthesised PCR products (15).

The reaction product was analysed by gel electrophoresis. Reaction products were mixed with Orange G loading dye (1:0.3 v/v) and run on a 2% agarose gel prepared with 0.5 x TBE containing 0.5mg/ml ethidium bromide. A Mini HE 33/Max HE 99V Horizontal Submarine Unit was used for casting and electrophoresis (Hoefer Scientific Instruments, Pharmacia Biotech). The product was run through the gel along with a suitable DNA marker, at a constant voltage (100V) in 0.5 x TBE running buffer. On completion, the gel was viewed under ultraviolet light and photographed.

3.4. *In situ* hybridization

The localization of TIMP-1 during the early stages of bone healing was observed using non-radioactive *in situ* hybridization on paraffin embedded tissue sections. Grafts and adjacent host bone were harvested on days 2 through to 9 and day 14 post-grafting and probed with both sense and anti-sense TIMP-1 DIG labelled riboprobes. Anti-sense and sense riboprobes were prepared by digesting the linearized plasmid with BamHl (sense) or XhoI (anti-sense). Using EcoRV (New England Biolabs), 100µl reaction mixtures were prepared for each probe containing 10 units of enzyme and 14µl linearized plasmid DNA in the relevant buffer: NEBuffer 2 plus BSA for XhoI and NEBuffer BamHl (150mM NaCl, l0mM Tris.HCl, l0mM $MgCl_2$, 1mM DTT (pH 7.9 @ 25°C) plus BSA (l00µg/ml) for BamHI. Fragments were amplified by PCR. A 200µl reaction mix using T7, SP6 and TIMP-1 anti-sense and sense primers (Table 2) were prepared.

RNA Labelling was performed by *In vitro* Transcription of DNA with Digoxigenin (DIG) RNA Labelling Mixture following a modified version of Boehringer Mannheim's protocol (16).

The hybridization buffer was warmed to 55°C before being used to dilute the riboprobe 1:9 (v/v). The freshly prepared riboprobe (55°C) was added to the sections as for pre-hybridization buffer and incubated overnight at 55°C. The sections were then transferred to TNE buffer at 37°C for 20 minutes before immersing in 20µg/ml RNaseA at 37°C for 30 minutes to remove any unhybridized probe from the sections. The hybrid was protected from digestion by the high salt concentration of the TNE buffer. Indirect detection of the probe was achieved by covering each section with polyclonal antibody against DIG conjugated to alkaline phosphatase (1:250 diluted in Buffer II with 5% NSS). The sections were kept in the dark until a purple/brown color developed. When the desired signal:noise ratio was observed the sections were immersed in stop buffer (20mM Tris HCl, pH 7.5 plus 0.5 mM EDTA) for 5 minutes to prevent the reaction from developing further. Kaiser's glycerol jelly was used to mount the sections.

4. RESULTS

Successful isolation of mRNA from the grafts was demonstrated by the presence of the housekeeping gene, GAPDH, which was chosen as its expression level remains fairly constant regardless of physiological conditions (17).

4.1. TIMP-1 Expression in Normal Adult Rabbit Bone

Total RNA extracted from samples collected on the day of grafting (day 0) did not contain mRNA for TIMP-1 (Figure 1a and b lane 2). RNA was successfully extracted since a 239bp band corresponding to GAPDH was observed in both the endochondral (Figure 1 lane 1) and intramembranous (Figure 1 lane 1) bone.

4.2. The Temporal Expression of TIMP-1 mRNA in Endochondral Bone Grafts

The temporal expression of TIMP-1 in endochondral bone grafts was seen to differ significantly from that of intramembranous bone grafts (Figure 1). In endochondral bone, TIMP-1 was expressed on days 1, 2 and 3 followed by a period of absence until day 8 and was present in all the remaining time points (Figure 1). Faint expression was noted on day 7 (1 out of 5 samples).

4.3. The Temporal Expression of TIMP-1 mRNA in Intramembranous Bone Grafts

In the case of intramembranous bone grafts TIMP-1 expression was not observed until day 6 and it was then expressed continuously through to day 11 and day 14 (Figure 1). Faint expression was observed once on day 5 (1 out of 6 rabbits) and also once on day 2 (1 out of 8 rabbits).

4.4. *In situ* Hybridization

Results revealed TIMP-1 to be present within the graft, interface and surrounding host bone. As with the RT-

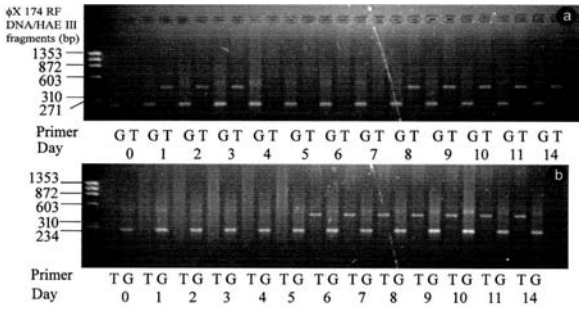

Figure 1. RT-PCR of total RNA extracted from autogenous endochondral (a) and intramembranous (b) bone grafts during the early stages of bone graft healing in the adult rabbit (days 0 to 11 and day 14). PCR products were resolved on a 2% agarose gel with timp-1 (t) giving bands at 425bp and GAPDH (g) giving bands at 239bp.

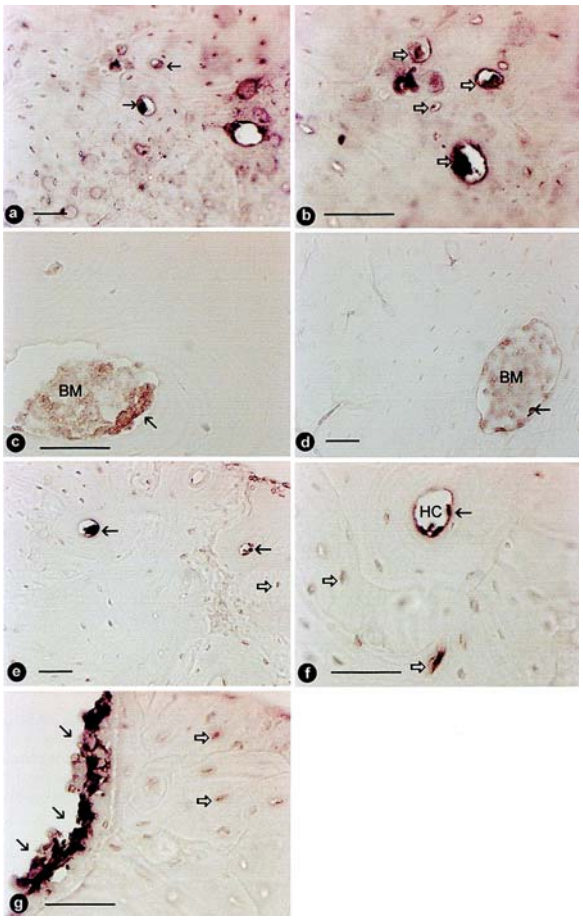

Figure 2. *In situ* hybridization of TIMP-1 mRNA expression in endochondral bone grafts on days 2 (a, b), 3(c), 5(d) and 7(e, f, g) post-grafting. Arrows indicate TIMP-1 mRNA expression within osteocytes (⇒) and bone lining cells (→). BM=bone marrow; HC=Haversian canal. Scale bar represents 40 μm.

PCR results the temporal expression was found to be different for defects grafted with endochondral bone as compared to those grafted with intramembranous bone.

4.5. *In situ* Hybridization in Endochondral Bone Grafts

In situ hybridization results for defects grafted with endochondral bone revealed TIMP-1 to be expressed within the grafts on days 2, 3, 5, 7, 8, 9 and 14 (Figures 2 and 3). Staining was present in bone lining cells covering the edge of the grafts (Figure 2g) as well as in cells located within Haversian canals (Figure 2f) and lacuna (Figure 2f). Expression of TIMP-1 was present throughout the study in cells covering the edge of the host bone and the newly forming matrix within the interface. Early expression was found to be weaker than that observed on later days.

Initial staining for TIMP-1 was observed within the grafts on days 2 and 3 (Figure 2a-c). However compared to other days, on day 3 TIMP-1 expression was sparse and weak, mainly being observed in Haversian canals near the edge of the graft (Figure 2c). On days 4 through to 6 there was no obvious staining within the endochondral grafts; with the exception of day 5 when staining was observed in cells lining a solitary medullar cavity as well as in osteocytes occupying adjacent lacuna (Figure 2d).

Days 7, 8, 9 and 14 showed strongly stained bone lining cells along the edge of the grafts as well as in cells occupying lacuna and Haversian canals (Figure 2e-g and 3). Although staining was present on day 7 it was only observed in bone lining cells covering parts, but not all, of the graft as well as in cells within Haversian canals and lacuna near the edges of the graft. Whereas on days 8, 9 and 14 stained cells were not restricted to areas around the edges of the grafts but were also present within the central regions. In all cases staining was more prevalent around the edges of the graft or in the vicinity of Haversian canals. Generally endochondral grafts were taken from the midshaft region of the diphyse but if they were collected near the metaphyseal area, medullar cavities were sometimes observed. In these cases staining was apparent in bone lining cells near the bone marrow (Figure 2c and d). Bone lining cells covering new bone within the interface and the host bone also stained for TIMP-1 (Figures 3a & c; 4a & e; 5a & d and 6a & d). Stronger staining was apparent when TIMP-1 was detected within the graft as well.

4.6. *In situ* Hybridisation in Intramembranous Bone Grafts

Intramembranous Bone Grafts Defects grafted with intramembranous bone revealed TIMP-1 to be expressed within the grafts on days 5, 6, 7, 8, 9 and 14 (Figure 7 and 8). No obvious staining was present on days 2, 3 and 4 although TIMP-1 expression was observed in the interface and host bone, to varying degrees at all time points (Figures 3b & d, 4b & f, 5b & e and 6b, c & e). On day 5, expression was very sporadic within the graft, appearing in the odd cell lining the bone marrow spaces (Figure 7a). By day six staining within the graft was intense and was observed in the majority of cells lining the bone marrow spaces as well as in cells lining the edges of the bone graft (Figure 7 and 8). Expression was very similar in both the host bone and graft bone adjacent to the interface on day 7 (Figure 5b). Days 8, 9 and 14 showed the most intense staining for TIMP-1 within the grafts (Figure 8).

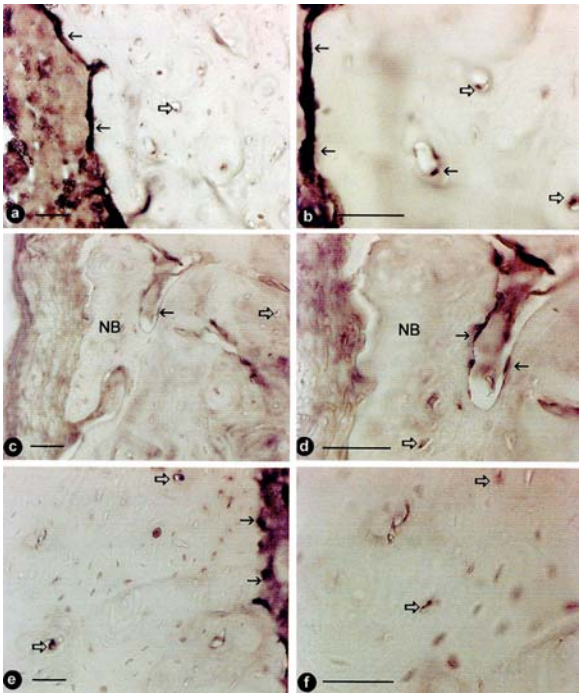

Figure 3. *In situ* hybridization of TIMP-1 mRNA expression in endochondral bone grafts on days 8 (a, b), 9(c, d), and 14(e, f) post-grafting. Arrows indicate TIMP-1 mRNA expression within osteocytes (⇒) and bone lining cells (→). NB=new bone. Scale bar represents 40 μm.

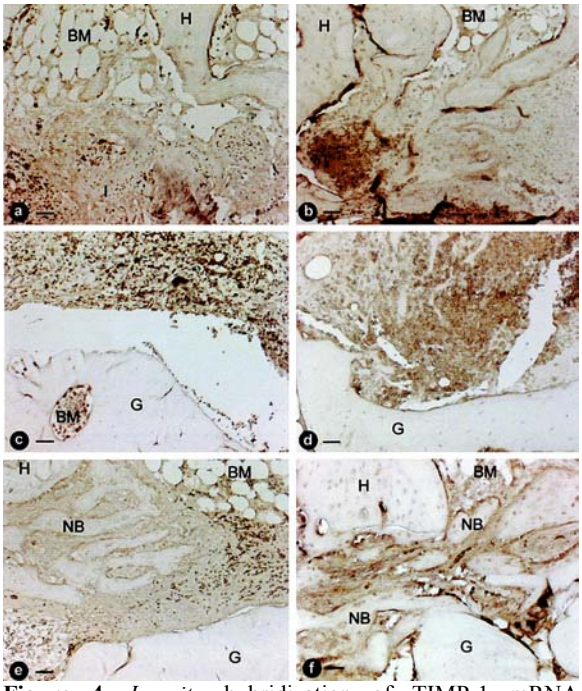

Figure 4. *In situ* hybridization of TIMP-1 mRNA expression within the host-graft interface on days on days 5 (a, b, c, d), 6(e, f) post-grafting. a, c, and e=defects grafted with endochondral bone; b, d and f=defects grafted with intramembranous bone. BM= bone marrow; G=graft; H=host; NB=new bone. Scale bar represents 40 μm.

Staining was apparent in osteocytes resident within the graft as well as in bone lining cells covering the outer surface of the graft (Figure 8a) and medullar cavities within the graft (Figure 8b, g and h). Osteoblasts lining the newly forming bone within the interface were all stained along with the hosts bone lining cells (Figures 4b & f and 5b & e).

5. DISCUSSION

The localization of TIMP-1 mRNA in cells within the grafted bone is an indication of an active participation of the bone graft in the integration of the grafted bone with the host bone. The bone graft undergoes resorption and releases different cytokines and BMPs to help with its integration with the host bone.

In defects grafted with endochondral bone, TIMP-1 mRNA expression was first observed in the graft bone on days 1 to 3 followed by a period of absence (Figure 1a, 3a and c). The appearance of TIMP-1 in the first three days means the amount of MMPs is decreased and the resorption is decreased. During this period the inflammation of the surgical insult is taking place where there is an influx of blood into the defect area as well as an initial inflammatory response. Cells brought into the defect area along with the blood include osteoclast precursors, inflammatory cells and undifferentiated mesenchymal cells. The macrophages engulf debris and release cytokines for inflammation (13). This study showed that when endochondral bone was grafted into skull defects, an inflammatory response and blood clot formation occurred on days 1 and 2 after grafting and activated macrophages were identified by day 3 (18).

After day 3, TIMP-1 expression disappeared on days 4, 5, 6 and 7 (Figure 1a). Chow and Rabie (19) reported that blood vessel invasion into endochondral bone, grafted into the same experimental model as the current study, occurred on day 4 post-grafting and reached a peak by day 6. The lack of expression of TIMP-1, in this study, coincides with the neovascularisation of the defect grafted with endochondral bone. Neovascularisation requires the action of the MMPs to breakdown the extracellular matrix to allow for endothelial cell migration. In addition, MMP also breaks down the bone graft, allows the release of cytokines like TGF-β I and II, numerous BMPs, PDGF and FGF for osteogenesis (20). This initial period of graft resorption would therefore be expected to occur along the edges of the graft in contact with the blood. Using *in situ* hybridisation, positive staining for TIMP-1 was indeed observed within bone lining cells situated around the edge of the graft although staining was not continuous around the whole of the graft. Cells lining the Haversian canals and osteocytes occupying adjacent lacuna also expressed TIMP-1 (Figure 3a and b).

On day 8, RT-PCR (Figure 1a) revealed that TIMP-1 expression reappears within the endochondral bone grafts and remains throughout the remaining studied time points (days 9, 10, 11 and 14). *In situ* hybridization carried out on days 8, 9 and 14 showed that TIMP-1 was expressed

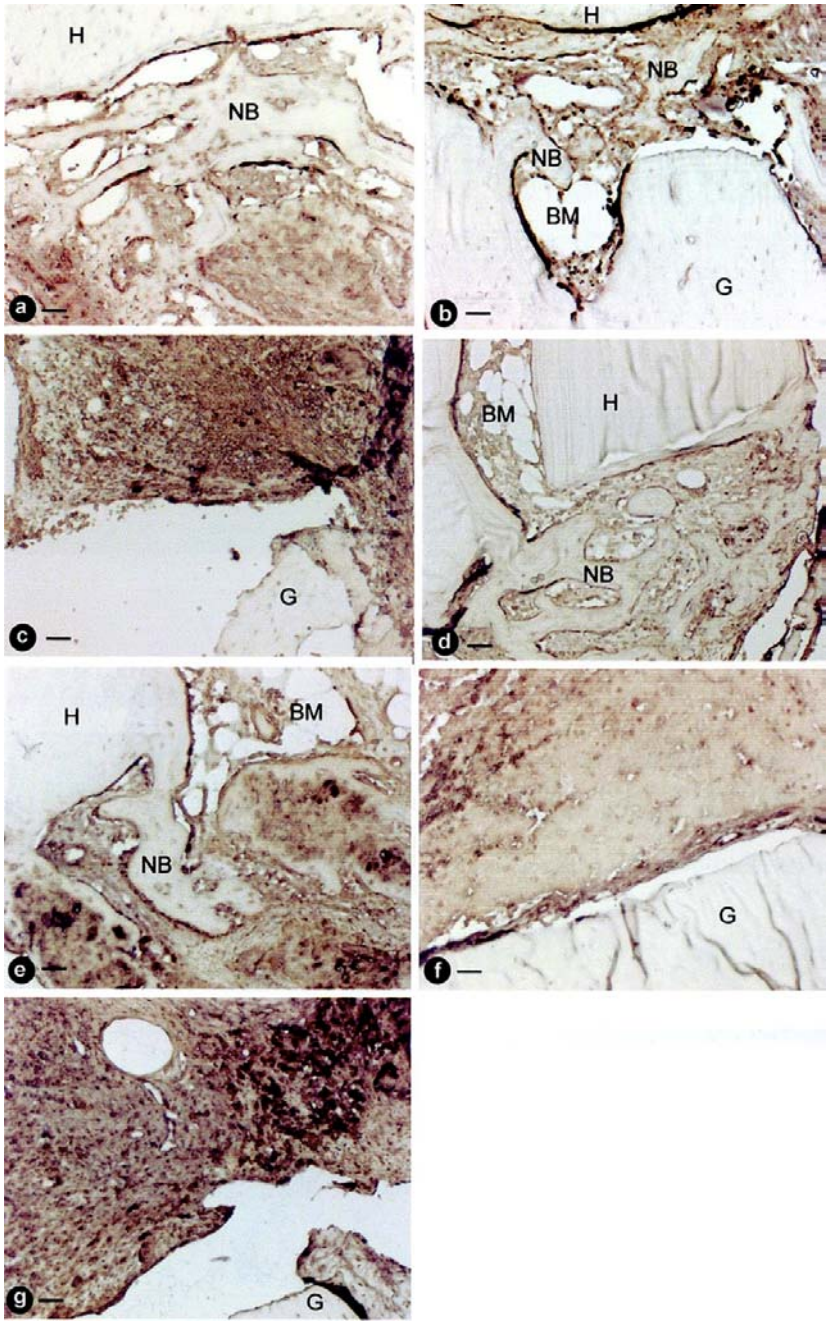

Figure 5. *In situ* hybridization of TIMP-1 mRNA expression within the host-graft interface on days on days 7 (a, b, c) and 8(d, e, f, g) post-grafting. a, c, d and f=defects grafted with endochondral bone; b, e and g=defects grafted with intramembranous bone. BM= bone marrow; G=graft; H=host; NB=new bone. Scale bar represents 40 μm.

strongly throughout the grafts (Figure 3). Staining for TIMP-1 was found in bone lining cells covering the graft as well as within cells lining Haversian canals and osteocytes occupying lacuna within the graft. The MMPs are therefore suppressed. This expression of TIMP-1 on day 8 after grafting coincides with osteogenesis at the interface between endochondral bone grafts and host bone. In a similar model as the current study, we reported that during endochondral bone graft healing osteoblast differentiation and endochondral ossification occurred on day 7 post-grafting (18). Therefore, the expression of the TIMP-1 on days 8 to 14 (Figure 1a and 3) is related to new bone formation.

In defects grafted with intramembranous bone, TIMP-1 mRNA expression was not observed in the graft

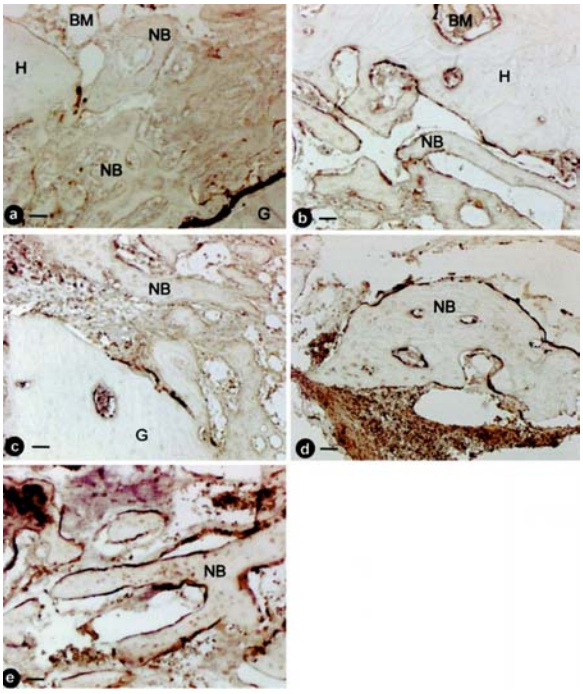

Figure 6. *In situ* hybridization of TIMP-1 mRNA expression within the host-graft interface on days on days 9 (a, b, c), 14 (d, e) post-grafting. a and d=defects grafted with endochondral bone; b, c and e=defects grafted with intramembranous bone. BM= bone marrow; G=graft; H=host; NB=new bone. Scale bar represents 40 μm.

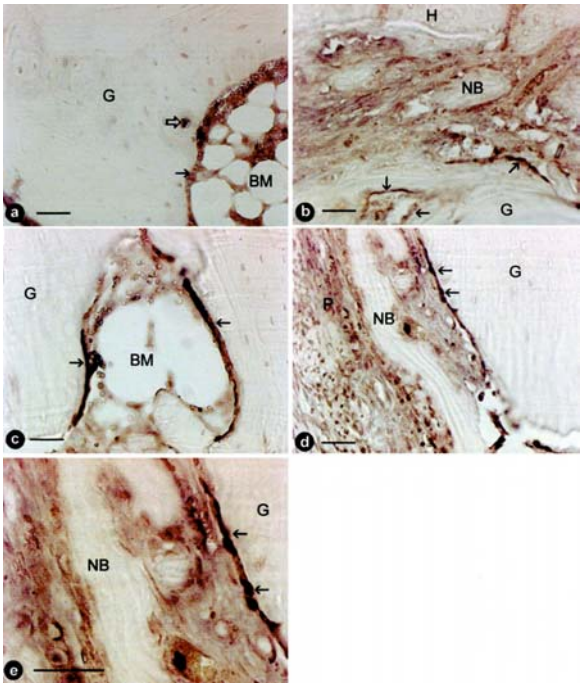

Figure 7. *In situ* hybridization of TIMP-1 mRNA expression in intramembranous bone grafts on days 5 (a), 6 (b) and 7 (c, d, e) post-grafting. Arrows indicate examples of TIMP-1 expression within osteocytes (⇒) and bone lining cells (→). G=graft; NB=new bone. Scale bar represents 40 μm.

bone from day 1 to day 5 (Figure 1b), *in situ* hybridization also revealed the absence of TIMP-1 within this time period.

Kusiak *et al.* (21) showed that intramembranous bone, when used as a graft material, undergoes rapid revascularisation when compared to endochondral bone grafts. The fact that TIMP-1 is not expressed by intramembranous bone grafts during the initial period of healing but is expressed by endochondral bone grafts suggests that the embryonic origin of the bone does influence the healing process in some way. TIMP-1 mRNA was not detected in the graft on days 1 through to 5 supports the need for MMP activity for the revascularization of the defect area. This agrees with the work by Chay and Rabie (22) where capillary budding was first observed in the host endothelial cells on day 3. The rate of vascular invasion reached a peak on day 4 after which it progressed at a decreasing rate until day 7. As well as being involved in the budding of the capillary cells, MMPs are also required to breakdown the surrounding extracellular matrix to allow for the migration of the small blood vessels observed on days 4 to 7.

Expression of TIMP-1 mRNA was observed by both RT-PCR (Figure 1b) and *in situ* hybridization studies (Figure 7 and 8) on days 6 to 9 and on day 14 within intramembranous bone grafts. Using RT-PCR analysis the expression of TIMP-1 mRNA was also observed on days 10 and 11.

In situ hybridization demonstrated the presence of TIMP-1 mRNA within the graft starting on day 6, staining was observed in the majority of cells lining the bone marrow spaces as well as in cells lining the edges of the bone graft. Days 8, 9 and 14 showed the most intense staining for TIMP-1 (Figure 8). The presence of TIMP-1 on this day means that any MMP activity within the defect area will be reduced or completely inhibited. By decreasing the amount of active MMPs present there would be a corresponding decrease in both the rate of vascularization and in the rate of bone resorption. Healing of intramembranous bone grafted into rabbit skull defects also showed that osteogenesis occurred on day 6 (Figure 7b). These results indicate that TIMP-1 was only expressed when an adequate blood supply had been established within the defect, which in turn ensures the initiation of osteogenesis. Once osteogenesis is underway TIMP-1 is expressed in order to protect the newly formed bone matrix from excessive resorption thus enabling complete integration of the graft with the host bone. By day 6, when TIMP-1 was also being expressed, new bone matrix could be observed throughout the interface (Figure 7) and was fully integrated with both the host and graft bone by day 14 (Figure 6).

6. CONCLUSION

The fact that the temporal expression of TIMP-1 was different in endochondral and intramembranous bone

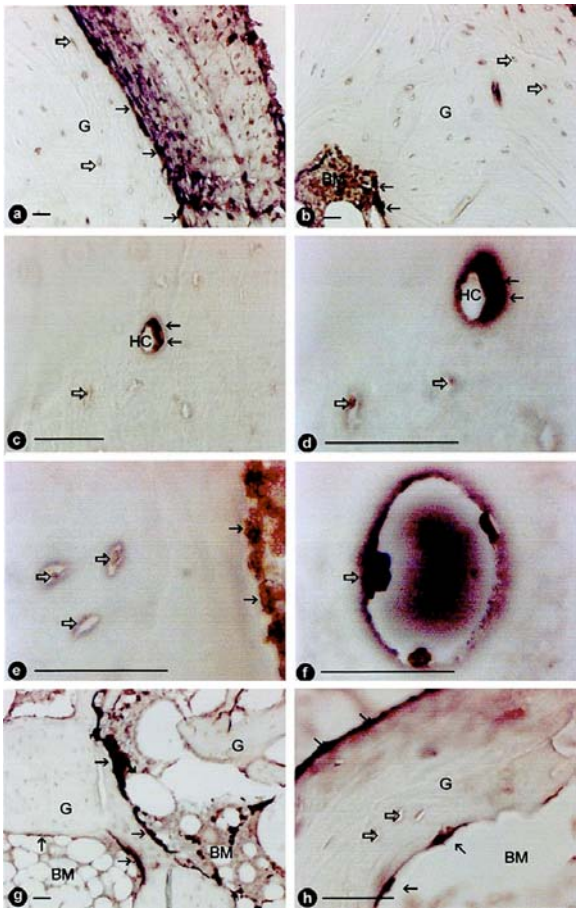

Figure 8. *In situ* hybridization of TIMP-1 mRNA expression in intramembranous bone grafts on days 8 (a, b, c, d), 9 (e, f) and 14 (g, h) post-grafting. Arrows indicate TIMP-1 expression within osteocytes (⇒) and bone lining cells (→). BM= bone marrow; G=graft; HC= Haversian canal. Scale bar represents 40μm.

grafts indicates that it may have a direct effect on the overall healing of these grafts due to its effect on revascularization and resorption.

Bone grafts play an important role in influencing the healing process mediated by the host tissues. The earlier expression of TIMP-1 by endochondral bone grafts, could be the reason for the delayed vascularization while the expression of TIMP-1 by the intramembranous bone grafts, at a later stage could allow earlier vascularization of the intramembranous bone grafts.

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