Sinus Grafting Using Recombinant Human Tissue Factor, Platelet-Rich Plasma Gel, Autologous Bone, and Anorganic Bovine Bone Mineral Xenograft: Histologic Analysis and Case Reports

Pierre Philippart, MD, LDS[1]/Valéry Daubie, Ir [2]/Roland Pochet, PhD[3]

Purpose: *The purpose of this study was to analyze healthy bone formation by means of histology and immunohistochemistry after grafting with a mixture of autologous ground calvarial bone, inorganic xenograft, platelet-rich plasma (PRP), and recombinant human tissue factor (rhTF).* ***Materials and Methods:*** *Maxillary sinus floor augmentation was performed on 3 patients by grafting with 5 to 10 mL of a paste consisting of autologous powder from calvarial bone (diameter < 1 mm), 50% v/v anorganic bovine bone mineral xenograft (PepGen P-15, a new tissue-engineered bone replacement graft material), PRP (1.8×10^6 platelets/mm^3 plasma), and about 1 µg rhTF. Six and 10 months after grafting, bone cores were extracted for implant fixation and analyzed.* ***Results:*** *Histology demonstrated a high degree of inorganic xenograft integration and natural bone regeneration. Both the xenograft and newly synthesized bone were colonized with osteocytes and surrounded by osteoblasts. Six-month-old bone cores demonstrated a ratio of synthesized bone to xenograft particles ratio of 0.5, whereas 10-month-old cores demonstrated a ratio of 2. A low degree of inflammation could also be observed using S100A8 immunohistochemistry.* ***Discussion:*** *Autologous grafting in edentulous patients is a complex procedure; the successful substitution of synthetic analogs for ground bone is a major challenge.* ***Conclusion:*** *In this investigation, it was shown that inorganic xenograft in the harvested bone paste could be safe for patients and had high bone regeneration capacity over time. The sinus graft showed intense bone formation 6 months after grafting and a further increase in bone growth 10 months after grafting.* Int J Oral Maxillofac Implants 2005;20:274–281

Key words: *angiogenesis, bone regeneration, immunohistochemistry, inflammation markers, S100 proteins*

Several experiments have investigated various aspects of autogenic and xenogenic bone graft incorporation to optimize graft survival and volumetric maintenance in sinus lifting procedures.[1,2] Autogenous bone has always been considered the "gold standard" because of its osteoinductive potential, compatibility, and effective therapeutic results. In clinical cases with severe maxillary bone atrophy (Cawood and Howell class V or VI),[3] a large amount of grafting material is required (about 10 mL for bilateral sinus augmentation). Harvesting autogenous bone graft is an essential part of treatment. Common donor sites are the mandible, the iliac crest, and the skull.[4,5] For extensive reconstructions, iliac bone is most commonly used. However, several disadvantages are associated with these treatment modalities. The morbidity of this harvest site is a real concern. Many patients from whom iliac bone was harvested have experienced a long period of postoperative pain and discomfort at their hips.[6] Another disadvantage is late bone resorption. The search for a graft material other than iliac bone is therefore still underway. In

[1]Head, Department of Oral and Maxillo-Facial Surgery, Bracops Public Hospital, Brussels, Belgium.
[2]Researcher, Laboratory of Histology, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium.
[3]Professor, Laboratory of Histology, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium.

Correspondence to: Dr Roland Pochet, Laboratory of Histology, Faculty of Medicine, Université Libre de Bruxelles, 808 route de Lennik CP620, B-1070 Brussels, Belgium. Fax: +322 5556285. E-mail: rpochet@ulb.ac.be

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Table 1 Patient and Implant Characteristics

Patient	Gender	Birth date	Graft type	Implants			Time between grafting and biopsy (mo)	Time implants in function (mo)
				No.	Type	Width (mm)		
DB	M	12-08-1948	Unilateral	2	Frialit II*	4.5 (1) 5.5 (1)	6	3
WM	F	09-12-1941	Bilateral	9	Xive*	3.8 (7) 5.5 (2)	10	3
CJ	F	05-03-1945	Unilateral	3	Frialit II*	3.8 (2) 5.5 (1)	6	3

*Dentsply Friadent Ceramed.

maxillofacial surgery, bone grafts from the skull (calvarial split bone grafts) are commonly used for the reconstruction of large bone defects of the midface[7] and seem ideal for the reconstruction of zygomatic and orbital defects. Postoperative bone graft resorption has been minimal.

Initially the authors used the conventional harvest procedure: Only the outer cortex was osteotomized to the shape and size desired (3 cm length, 1 cm width) and harvested from the parietal skull using chisels. The rate of neurologic complication (dural tears, epilepsy) was about 0.5%, with local complications in the range of 3% to 5%.[8] To minimize donor site morbidity related to the harvest procedure, a round bur (8 mm diameter) was used to harvest the bone of the outer cortex. To obtain a sufficient quantity of powder, bone substitutes were added to autogenous bone.[9–11] Many surgeons use materials such as inorganic bovine bone mineral, β-tricalcium phosphate, coral hydroxyapatite, or bioactive glasses, which are only osteoconductive.[11–16] The authors, however, used PepGen P-15 (Dentsply Friadent Ceramed, Lakewood, CO), a new bone graft material that is a combination of both the mineral component of bovine bone (OstoeGraf/N-300; Dentsply Friadent Ceramed) and a peptide, P-15, representing the cell-binding domain of type I collagen. For bone regeneration and growth, differentiating factors as well as stem cells and matrix are needed. In the physiologic healing process, platelets are the natural source of growth factors and the fibrin network (the natural matrix), and marrow stem cells are the natural progenitors of osteocytes.[17] In their alpha granules, platelets store platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, and epidermal growth factor, which are released after activation.[18–21]

In a previous study,[22] the successful use of a mixture of recombinant human tissue factor (rhTF), autologous platelet-rich plasma (PRP), and particulate autologous calvarial bone was demonstrated. In the present investigation, the mixture was modified by substituting 50% of the calvarial bone with a commercially available inorganic bovine bone material, and bone and PepGen osseointegration were analyzed morphologically. It was found through this study that S100A8 appeared to be a good marker for PepGen and allowed researchers to follow the osseointegration of the xenograft. Originally, S100A8 immunohistochemistry was chosen because S100A8 is the most abundant protein within activated neutrophils. It is released by phagocytes and involved in the migration of phagocytes,[23] making it a potential marker for inflammation.

S100 proteins are calcium-binding proteins, which possess 2 calcium-binding domains of the EF-hand type. A total of 20 proteins have been now assigned to the S100 protein family.[24] They have a relatively small molecular mass (around 10 kDa) and are involved in cell differentiation, cell cycle progression, and regulation of kinase activities and cytoskeletal-membrane interactions.[25,26] Specific S100 proteins that presented interesting properties for bone immunohistochemistry were used; eg, S100A8 and S100A9, which are proinflammatory proteins,[27] S100A4, which has a negative effect on mineralization and osteoblast differentiation,[28] and S100A6, which is expressed within fibroblasts.[29]

MATERIALS AND METHODS

Three patients (Table 1) with uni- or bilateral severe atrophy of the maxillary alveolar process, diagnosed by panoramic radiographs and by Denta-Scan Asteion 4 captors (Toshiba, Brussels, Belgium) (Figs 1a and 1b), were included in this study. One patient was completely edentulous, and the other 2 had unilateral

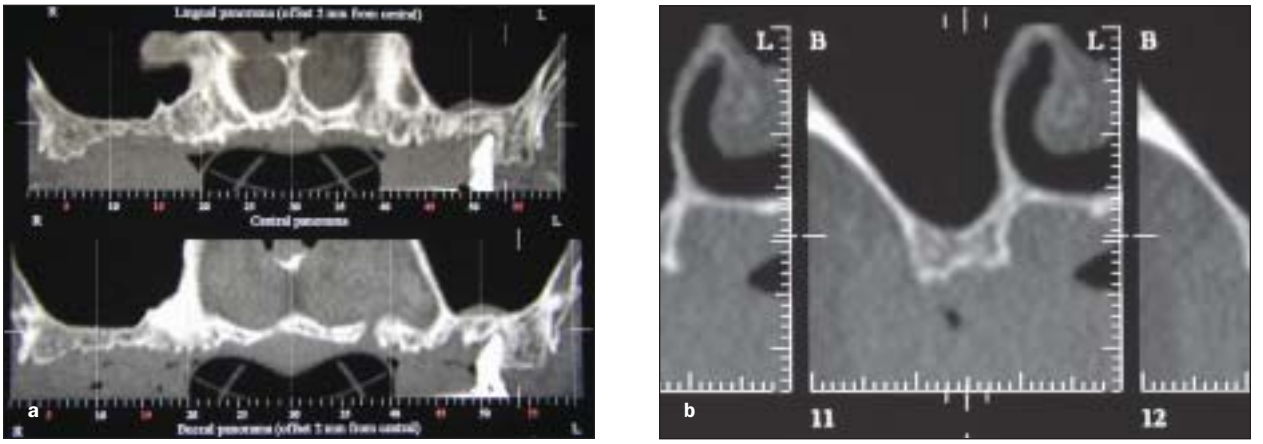

Fig 1 Maxillary computerized tomographic (CT) scan of an edentulous patient who presented with complete bilateral maxillary pneumatization (Class VI Cawood) before surgical reconstruction.

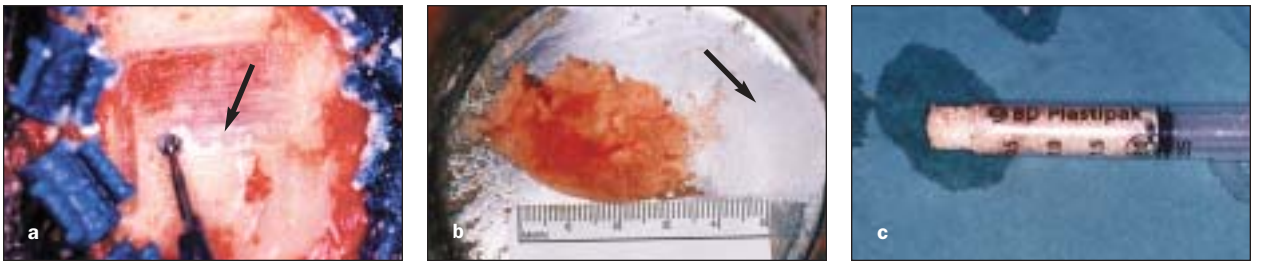

Fig 2 *(a)* Parietal cortical bone harvesting with rotating spherical bur, producing bone powder *(arrow)*. *(b)* Inorganic xenograft *(arrow)* and bone powder were combined in equal quantities. *(c)* Syringe filled with 1 aliquot of bone "paste" made of autologous bone powder, inorganic xenograft, PRP, and rhTF.

losses of premolar and molar segments. The patients had less than 4 mm maxillary bone height (range, 1 to 3 mm) and were treated with an augmentation procedure (ie, filling the defects with bone paste) and delayed implant placement. The study was approved by the Regional Ethical Research Committee (Bracops, IRIS SOUTH Public Hospitals, Brussels, Belgium).

Calvarial Bone Graft Harvesting

Cortico-parietal skull grafts were harvested under general anesthesia without antibiotic prophylaxis. The harvesting was performed using a spherical bur (6 mm diameter) (Fig 2a); a thin powder of cortical parietal bone was obtained. The bone powder (3 to 5 mL) was mixed with an isotonic solution (1 mL) (sodium chloride [NaCl] 0.9%) to avoid dryness. The galea and muscle were sutured in 1 layer; the scalp was sutured in a second layer with a compressive bandage.

Maxillary Sinus Augmentation Procedure

Bone Paste Preparation and Filling Procedure. PepGen P-15 (bovine bone–derived hydroxyapatite particles with a synthetic peptide) was added to the bone powder in a 50:50 ratio to reach a final volume of 5 to 10 mL (Fig 2b). Five milliliters of PRP was prepared by the SmartPReP procedure (Harvest Technologies, Plymouth, MA) and added to the bone mixture. To induce platelet activation, make the mixture easier to handle, and hinder the migration of particles, which could create holes in the membrane, 1 µg of rhTF (Innovin Lyophilized Thromboplastin; Dade Behring International, Paris, France) was dissolved in 2 mL of sterile water and added to the granulated solution to create a "gel paste." Fibrin clot was apparent within less than 1 minute.[30] The paste was placed on sterile gauze and loaded in the syringe to be applied according to the need for a sinus lift procedure in the maxilla (inlay bone graft) (Fig 2c). The gel was easy to handle and prevented graft material from migrating or penetrating holes in the membrane. The wounds were sutured with silk sutures. Computerized tomographic scans were obtained 2 months after the surgical filling procedure (Figs 3a and 3b).

Surgical Technique. Following a supracrestal incision with mesial and distal releasing incisions extending well up into the buccal fold, a full-thick-

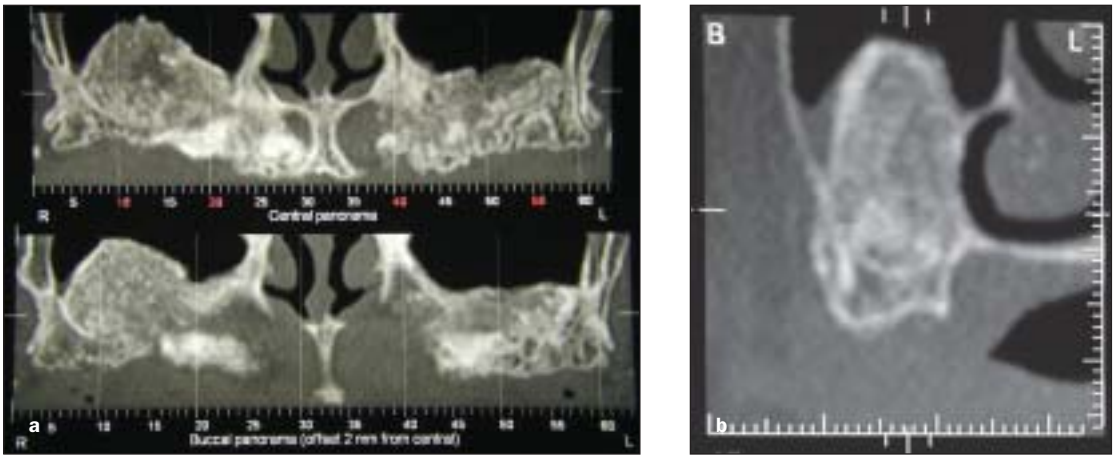

Fig 3 *(a)* A CT scan obtained 2 months after grafting (compare with Fig 1a). *(b)* An offset view that shows the homogenous filling of the whole sinus cavity *(frontal view).*

ness mucoperiosteal flap was reflected. An oval-shaped osteotomy (approximately 20 mm wide and 10 mm high) was outlined with a round bur on the lateral aspect of the alveolar ridge under copious sterile water irrigation. The sinus membrane was reflected apically and medially. Care was taken not to lacerate the sinus membrane during this elevation procedure. The graft mixture was packed layer by layer to fill the sinus hole. The wounds were sutured with resorbable sutures.

Biopsy Procedure

Bone core extraction and implant placement were carried out after a healing period of either 6 or 10 months. The bone core was extracted with a trephine (3 to 4 mm in diameter) in an axial direction into the grafted area. Implants were placed at the site of the bone core extraction. The average length of the bone cores was 12 mm (range, 8 to 14 mm).

Histologic Preparation

Bone cores were immediately immersed in buffered 4% paraformaldehyde fixative for 5 days. They were then decalcified from 3 to 24 hours, depending on bone density, in a solution made of 5% formic acid, 7% ammonium chloride, and 1.4% hydrochloric acid. The decalcified bone core was dehydrated in alcohol baths of increasing concentrations and included in paraffin. Sections 5 µm wide were applied on poly-L-lysine precoated slides (Sigma Aldrich, St Louis, MO).

Immunohistochemistry

A standard protocol was followed[31]; the rehydrated sections were first incubated in 3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes to inhibit internal peroxydase and rinsed in 50 mmol/L tris-buffered saline (TBS) solution (pH = 7.3). They were then incubated with 5% blocking serum (horse for a-S100A4 and A6, sheep for a-S100A8, A9, and B) in TBS for 30 minutes at 37°C to prevent unspecific binding of the secondary antibodies. The sections were then rinsed and incubated overnight at 4°C with polyclonal antibodies against respective S100 proteins diluted in TBS with 3% bovine serum albumin (BSA; Sigma, St Louis, MO): 1/2,000 for antibodies against S100A4 protein (Kinderspital Zürich, Zürich, Switzerland) and S100A8 and S100A9 proteins (Institut für Experimentele Dermatologie, Münster, Germany), and 1/10,000 for antibodies against S100A6 proteins (Kinderspital Zürich) and S100B proteins (SWant, Bellinzona, Switzerland). Sections were incubated with secondary polyclonal affinity purified antibody for 30 minutes at room temperature. Biotinylated anti-goat immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA) was used for antibodies against S100A4 and S100A6 proteins, and biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was used for antibodies against S100A8, S100A9, and S100B proteins. Finally, sections were stained using avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Laboratories), with diaminobenzidine/H_2O_2 (BioGenex, San Ramon, CA) as the chromogenic substrate.

Synthesized Bone/Xenograft Particles Ratio Calculation

Synthesized bone/xenograft ratio was based on a 2-dimensional area measurement. For each case, statistical evaluation was calculated from 20 pictures (n = 20)

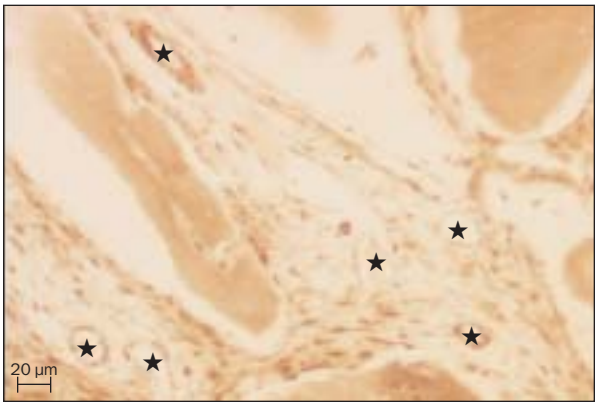

Fig 4 S100A6-immunohistochemistry of a bone core 6 months after grafting. Note the numerous blood vessels (indicated by ★).

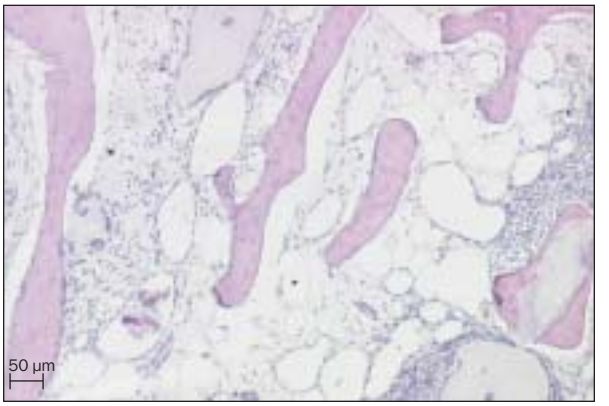

Fig 5 Section of a 10-month-old bone graft showing a typical medullar bone configuration (hematoxylin-eosin).

taken from 6 different slides. The slides were randomly selected from all available bone cores. For each picture, the areas covered by new synthesized bone and xenograft particles were determined using ImageJ 1.31 software (National Institutes of Health, Bethesda, MD). The mean was calculated for each picture, and standard deviation was calculated from all mean values.

RESULTS

The S100 immunohistochemical staining accentuated the structural and constitutive properties of the bone graft, as well as new bone properties of S100 proteins. The graft material, inorganic xenograft, was well integrated in the connective tissue, which appeared organized, with dense vascularization including both veins and arteries (Fig 4). Ten months after grafting, the connective tissue morphology resembled that of natural medullar bone; it was scattered with vacuoles (Fig 5). The newly regenerated natural bone as well as the xenograft occupied a large core area. In most cases, regenerated bone included inorganic xenograft fragments completely surrounded by new bone (Fig 6a). The ratio of new bone to xenograft increased from 1:2 for 6-month-old bone cores to 2:1 for 10-month-old ones (Figs 6b and 6c). Osteocytes, which appeared S100B immunoreactive, were found in both new bone and xenograft (Figs 6d and 6e). Osteoblasts surrounding the new bone could be detected (Fig 6f), and osteoblasts and osteoclasts surrounding xenograft were also seen (Fig 6g) using S100A6 immunoreactivity. S100A8 and S100A9 immunohistochemistry showed a low presence of activated neutrophils and activated macrophages, which confirmed the lack of inflammation in the graft. Interestingly, anti-S100A8 and anti-S100A9 also specifically stained the inorganic xenograft and therefore could be used as good markers (Fig 6h).

DISCUSSION AND CONCLUSION

This study provides further morphologic data indicating that in humans, bone regeneration with osteoinduction can be safely achieved using a "bone paste" made of PRP gel with rhTF and a mixture of inorganic xenograft and autologous bone in a 50:50 ratio. Comparison between 6- and 10-month-old bone cores demonstrated that the xenograft was "replaced" by natural bone. Although the substitution mechanism is still unclear, detection of osteoblasts and osteoclasts in and around the xenograft material is in agreement with the following hypothesis: The matrix of the PepGen can be removed by the bone cells, which then reconstruct their new matrix scaffold. Safety has been improved by using rhTF as an exogenous protein, which avoids the usual concerns regarding adverse reactions to exogenous materials (eg, bovine thrombin), disease transmission, and immunogenic reactions.

The authors propose that another factor in the successful use of this graft material was the concomitant action of TF and PRP, which allowed fast clotting and avoided diffusion or dilution of the trapped growth factors. Indeed, TF induces the immediate clotting reaction and therefore maintains in situ growth factors at efficient concentrations. This is in agreement with previous studies, which have shown that quantifiably enhanced results were obtained when PRP[18–21] or TF[22] were added to the graft material compared to grafts performed without their use. Moreover, a protein scaffold of clot (composed of fib-

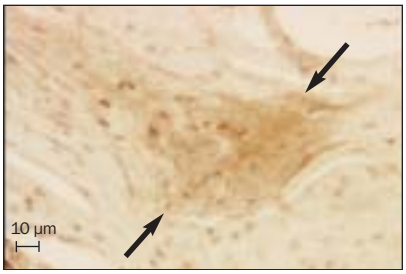

Fig 6a S100B immunohistochemistry of a bone core 10 months after grafting. Osteocytes appeared S100B-positive. Both bone and PepGen *(between arrows)* are visible and are intimately associated.

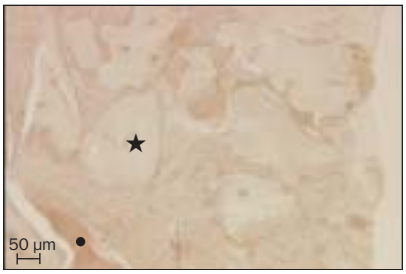

Fig 6b H&E staining of a bone core 6 months after grafting (star = anorganic xenograft, circle = new synthesized bone).

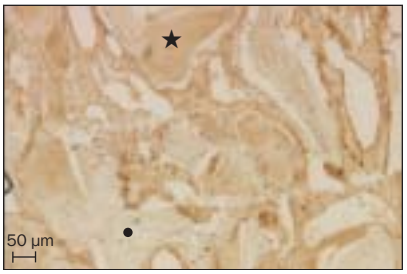

Fig 6c S100B immunohistochemistry of a bone core 10 months after grafting (star = anorganic xenograft, circle = new synthesized bone).

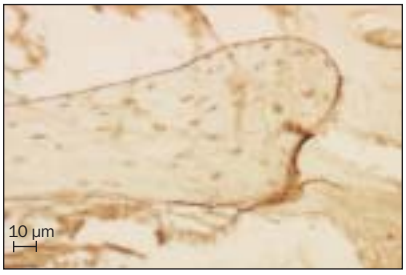

Fig 6d Immunohistochemistry of a bone core 10 months after grafting. S100B-positive osteocytes are visible within natural bone.

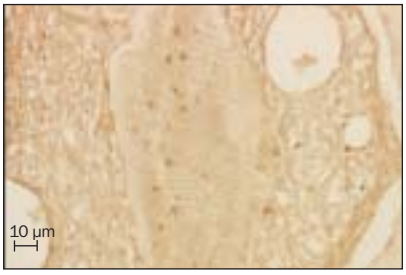

Fig 6e Immunohistochemistry of a bone core 10 months after grafting. S100B-positive osteocytes are visible within inorganic xenograft.

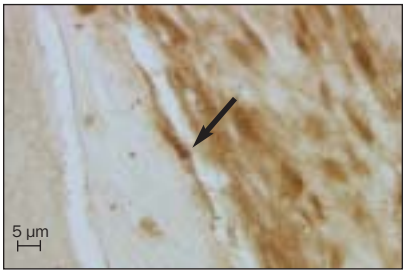

Fig 6f Immunohistochemistry of a bone core 10 months after grafting. S100A6-positive osteoblast *(black arrow)* in contact with bone.

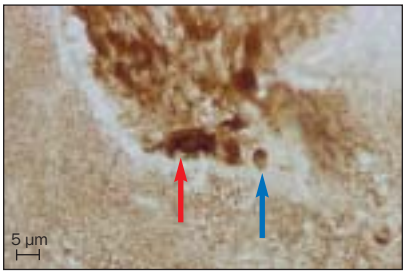

Fig 6g Immunohistochemistry of a bone core 10 months after grafting. S100A6-positive osteoclast *(red arrow)* and osteoblast *(blue arrow)* in contact with inorganic xenograft are visible as finely granular material.

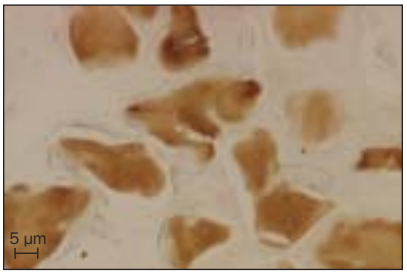

Fig 6h S100A8 immunohistochemistry of a bone core. All visible particles are xenograft particles.

rin, fibronectin, and vitronectin) is required for cell adhesion and cell differentiation,[32–36] which is another "added value" to the system. As we begin to decipher the TF mechanism of action, the following has been found.

TF and Coagulation Cascade

The TF known as thromboplastin is a transmembrane glycoprotein, which forms a complex with blood coagulation factor VII (FVII) and factor VIIa (FVIIa). However, it has recently been suggested that TF is present in human blood.[37] In the plasma, TF is mainly associated with circulating microvesicles.[37] This was confirmed by the presence of full-length TF in microvesicles acutely shed from the activated platelets. TF was observed to be stored in the alpha granules and the open canalicular system of resting platelets and to be exposed on the cell surface after platelet activation.[38] TF binding to DVII and DVIIa triggers their proteolytic activity.

TF: A Signaling Receptor

An exciting recent development in TF biology is the demonstration that this protein, a member of the cytokine receptor superfamily, can function as a signaling receptor.[30,39,40] Signaling via TF has now been documented in many different cell types including platelets, endothelial cells, keratinocytes and astrocytes,[41,42] which act through binding to FVIIa.[43,44] TF-FVIIa complex on human fibroblasts leads to activation of phospholipase C and enhanced platelet-derived growth factor BB–stimulated chemotaxis,[45] induces proinflammatory effects in macrophages,[46] and increases vascular endothelial growth factor production by human fibroblasts.[47] In the specific case of bone regeneration in humans, TF induces and enhances angiogenesis.[48–50]

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