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Bone allograft non-union is related to excessive osteoclastic bone resorption: A sheep model study

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Summary. Using a sheep femoral allograft model we have investigated the cellular and molecular mechanisms associated with non-union of bone allografts. Histomorphometric analysis revealed that allograft non-unions featured both marked increases in osteoclast (OC) numbers and total eroded bone surface as compared to allografts wich had undergone direct union. Three distinct cellular layers lying adjacent to the allograft bone surface were identified in all non-union cases. The outer or fibroblastic layer contained an abundance of fibroblasts and connective tissue. Circumscribing this layer was a band of synovial-like cells consisting mainly of large spindle-shaped mononuclear cells mixed with scattered round-shaped mononuclear cells. The third layer, which was directly juxtaposed to the allograft bone surface, consisted predominantly of multinuclear OCs which were positively identified by calcitonin receptor immunohistochemistry. Interestingly, *in-situ* hybridisation revealed that surrounding synovial-like cells in non-union allografts, expressed abundant gene transcripts for receptor activator NF-κB ligand (RANKL), a membrane bound factor critical for both the induction of OC activity and osteoclastogenesis. We propose that excessive bone resorption by host OCs contributes, at least partially, to the failure of bone allografts. The production of RANKL by synovial-like fibroblasts may be the driving force responsible for the elevated generation and activation of OCs. Based on such evidence novel therapeutic strategies for the treatment of non-union bone allografts using anti-bone resorbing agents may be devised.

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Introduction

Skeletal tissue allotransplantation in reconstructive surgery has increased steadily worldwide over the past decade. With the sole exception of blood, bone has become the most commonly transplanted tissue in humans. In the United States alone it has been estimated that over 220,000 bone grafts are implanted annually (Buck and Malinin, 1994). The culmination of an ageing population together with younger and more active patients undergoing hip replacement has increased the number and demand of impaction bone grafting in joint arthroplasty and limb salvage surgery (Harrington et al., 1986; Aro and Aho, 1993; Gie et al., 1993). Although bone transplantation has been used widely as the standard approach for reconstruction following excision of diseased bone, the pathogenesis underlying various complications that arise post-operatively are presently unclear. Among these, non-union and subsequent fracture or loosening is perhaps the major contributing factor responsible for failure of bone allografts (Mankin et al., 1983; Martin and Sutherland, 1993; Thompson et al., 2000). While non-union has been largely attributed to inadequate stability, compression or re-vascularisation of the graft, either alone or in combination, the nature of the cellular events that account for these phenomena remains poorly defined.

In this study, we established a model of bone allograft in sheep and used it to conduct complementary histological and immunohistochemical assays for precise cellular identification, while histomorphometric parameters of bone turnover were correlated with appropriate *in situ* hybridisation investigations. Our findings suggest that enhanced activation and recruitment of host OCs directly contributes to the non-

union of bone allografts and may in fact be the cellular mechanism underscoring bone allograft failure. Moreover, we propose that the elevated activation of OCs is related to the production of RANKL by synovial-like fibroblasts in non-union cases. Based on these data novel therapeutic strategies for the treatment of non-union bone allografts using anti-resorptives could be devised.

Materials and methods

Antibodies and reagents

Biotinylated swine anti-rabbit IgG and horseradish peroxidase (HRP)-conjugated strepdavidin were purchased from DAKO Corporation, CA, USA. Polyclonal rabbit antibodies directed against mouse calcitonin receptor (CTR) were kindly provided by Dr P.M. Sexton (Quinn et al., 1999). Non-radioactive Digoxigenin (DIG) RNA labeling kit was purchased from Boehringer Mannheim, Mannheim, Germany. All other reagents were purchased from Sigma, St Louis, USA unless stated otherwise.

Sheep femoral allograft models

Two year old male sheep (N=16) were used for the bone allograft experiments. All animal work was approved by an institutional ethics committee in compliance with the National Health and Medical Research Council (Australia) guidelines. Briefly, femurs were surgically removed from sex/age matched donor sheep in a sterile environment. Each femur was disarticulated at the knee, followed by removal of muscle by scraping the bone with an osteotome. A scalpel was used to cut the abductor tendons, the joint capsule removed from the neck of the femur, and the femoral head was dislocated anteriorly.

Femurs were promptly placed on ice within 1 hr after removal, frozen to at least -20°C within 4 hrs, and stored in a -70°C freezer within 24 hrs. Following this initial collection procedure, allografts were taken to ultra-low temperatures at –80°C and then hydrated under a high vacuum to remove resident bone marrow cells, which are considered to be immunogenic.

Intercalary allografts (8 cm in length) were used as reconstructions of diaphyseal or metaphyseal surgically produced defects in femurs of host sheep. The femoral intercalary allografts were performed as previously described in detail by Enneking et al. (1980). Following excision of the diaphyseal segments, reconstruction by osteo-synthesis of intercalary allografts was performed. The bone grafts were stabilized using intramedullary nails with compression screws. All experiments were performed by two qualified orthopaedic surgeons under identical conditions. Radiography was carried out every two weeks to monitor healing. Sheep were sacrificed eighteen weeks post- surgery, a period considered reasonable for union and healing to have occurred (Burchardt, 1983; Delloye et al., 1992).

Histology and histomorphometry

Appropriate samples (see below) from femurs of allograft recipient sheep were removed under sterile surgical conditions. Tissue samples were then fixed in 10% buffered formalin solution (BFS) for at least 48 hrs and then subjected to radiography. Tissue samples were sliced in a longitudinal direction and embedded in methylmethacrylate (for mineralised sectioning) or in paraffin (for decalcified sectioning). All tissue samples were processed and sectioned using established protocols in a routine histopathology laboratory. For histomorphometric analysis, longitudinal methyl-methacrylate sections of the junction between host and donor bone were used. A minimum of 1 cm in length of both host and allograft bone were included in each sample. Sections were either stained with Haematoxylin and Eosin (H&E) or Masson trichrome. Static parameters of bone turnover were assessed using the Osteomeasure™ semi-automated system. The dimensions of the measuring field were previously calibrated according to the Osteomeasure™ Documentation and User Guide (version 2.2)™. Graft sections were analysed field by field until a complete image of the graft was generated by the Osteomeasure™ system. Static histomorphometric parameters of bone turnover included osteoid surface/bone surface (OS/BS), eroded surface/bone surface (ES/BS), quiescent surface/bone surface (QS/BS) and osteoclastic surface/bone surface (Oc.S/BS) ratios. In addition, OC number, number of OCs per mm of eroded surface (N.Oc/E.Pm) and number of OCs per mm of graft bone surface (N.Oc/B.Pm) were also measured. All samples used for histological and histomorphometric analysis were deemed to be at equivalent stages of healing at 18 weeks.

Immunohistochemistry

OCs were readily identified using calcitonin receptor (CTR) immunohistochemistry. Prior to addition of primary antibodies, antigenic sites were unmasked by incubating sections in 10 µg/ml saponin in distilled water, for 30 min, followed by three washes in 0.2% saponin in PBS for 5 min. To ensure specificity, sections were then incubated in PBS containing 5% sheep serum, for 10 min, and washed three times in 0.2% saponin in PBS, for 5 min. Rabbit polyclonal CTR antibodies (Quinn et al., 1999) were diluted 1/50 in PBS containing 1% BSA, before being incubated with the tissue sections for 1 hr at room temperature, followed by 3x5 min washes in 0.2% saponin in PBS. To quench endogenous peroxide, sections were placed in 3% H_2O_2 in methanol for 15 min and then rinsed in PBS. Sections were incubated with biotylated swine anti-rabbit IgG, washed and colour was developed using HRP-conjugated streptavidin. Sections were then counterstained with

Gilles Haematoxylin and dehydrated before being mounted in DePeX and photographed under bright field microscopy.

In situ hybridisation

In situ hybridisation was used to assess the mRNA expression of receptor activator NF-κB ligand (RANKL). For this purpose, a cDNA fragment (486bp) of human RANKL was transcribed into DIG-labelled antisense riboprobes with T7 RNA polymerase using a DIG RNA labelling kit (Boehringer Mannheim). This fragment encodes an evolutionally conserved region of RANKL and thus was deemed suitable for the detection of mRNA transcripts in sheep. *In situ* hybridisation was performed according to previously described methods (Huang et al., 2000). The final concentration of each probe in the hybridisation solution was 0.3 ng/ml. RNAse treatment (100 µg/ml) prior to hybridisation served as a negative control.

Statistical Analysis

Data from histomorphometric measurement were analysed using the SPSS version 9.0. After confirming that data for each parameter was normally distributed, multivariate and univariate Analysis of Variance (ANOVA) was conducted to compare means for each outcome (union and non-union). A lack of fit test was conducted to ensure that the data fitted the ANOVA model. Finally, correlations between parameters were assessed by Pearson's test.

Results

Radiological and histological characterization of non-union bone allografts

Following allograft surgery, healing was monitored every two weeks by radiography. Little difference in healing was observable in radiographs 4-weeks post-surgery (data not shown). However, obvious differences in bone healing was evident by 18 weeks (Fig. 1), a period deemed sufficient for complete union and healing to have occurred (Burchardt, 1983; Delloye et al., 1992). Fig. 1 illustrates representative radiographs from sheep undergoing union and non-union healing at 6, 10, 16 and 18 weeks post-surgery. At 18 weeks, direct union can be clearly distinguished with good integration between host and allograft bone as depicted in segmental bone

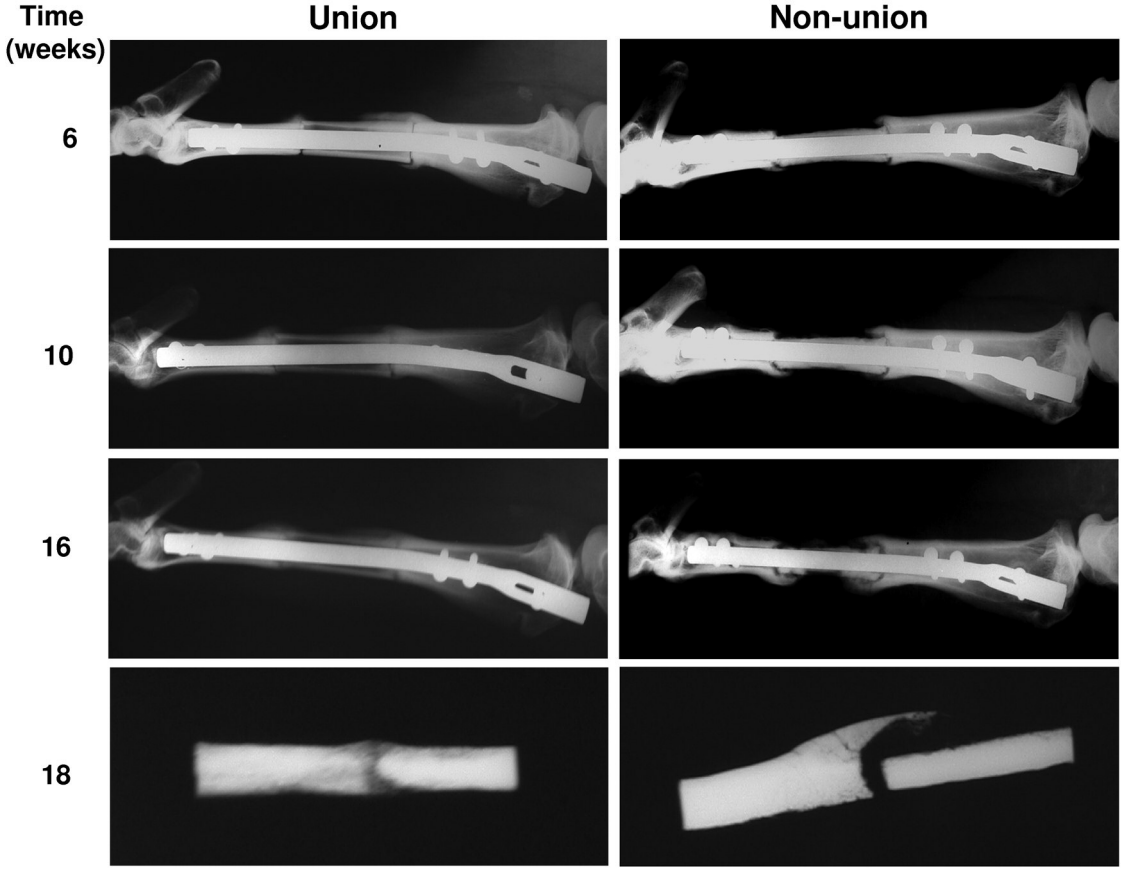

Fig. 1. Radiographs of femoral intercalary allografts at 6, 10, 16 and 18 weeks post-surgery. Union shows excellent integration between host and allograft bones and bone callus formation is clearly evident on the junction of the bone allograft by 16-18 weeks healing. Non-union shows massive osteolysis on the surface of the bone allograft and poor integration and callus formation at the junction. Note: 6-16 week radiographs were taken from living animals while radiographs at 18 weeks were taken from bone segments at the host-graft junction after harvesting.

radiographs. By comparison, non-union cases were typically characterized by a lack of integration between host and allograft bone. Strikingly, all non-union cases exhibited marked osteolysis in radiographs at the allograft-host bone junctions. From a total of 16 sheep which received bone allografts, 9 exhibited complete union whereas 7 allografts failed to unite and thus were classified as non-union cases at the end of 18 weeks.

To gain more detailed insight into the cellular mechanisms underlying bone allograft failure we next compared the histology of the allograft-host bone interface between union and non-union allografts. As expected, in those sheep which had undergone direct allograft union, bone formation by host osteoblasts could be observed directly on allograft surfaces (Fig. 2A). In some instances, osteoid had almost completely surrounded both the graft and host bone. In addition to this osteoid deposition, extensive internal remodelling was evident both within the allograft tissue as well as the new osteoid deposits. Comparatively, non-union allografts displayed little osteoid deposits with unique structural changes observed at the surface of the graft-host interface (Fig. 2B). The allograft surface was highly eroded, exhibiting numerous Howship's lacunae and an abundance of multinucleated OC-like cells overlying the graft surface (Figs. 2B, 3A). External to this multinucleate cell layer, a zone consisting mainly of polygonal-shaped cells with a synovial-like appearance mixed with round shaped mononuclear cells was discernable (Fig. 3B). The outermost layer was composed almost exclusively of vascularised fibrous tissue (Fig. 3C). Interestingly, there was little evidence of lymphocyte recruitment or inflammatory response in all non-union allografts examined.

To confirm that the observed multinucleated cell layer represented OCs and not reactive foreign body giant cells, we performed immunocytochemistry using antibodies against calcitonin receptor (CTR), a specific marker of mature OCs. As shown in Fig. 4A, all multinucleated cells associated with resorptive lacunae stained positive for calcitonin receptor confirming the identity of these cells as OCs. In contrast, adjoining synovial-like cells failed to show CTR immunoreactivity thus eliminating the possibility that these cells may be of OC lineage (Fig. 4B). Occasionally, a few round-shaped mononuclear cells scattered within the synovial-cell layer displayed weak to moderate CTR staining, possibly reflecting circulating OC precursors or pre-osteoclastic cells. Importantly, no appreciable immunostaining was observed in either the absence of the CTR serum or when cells were treated with pre-immune serum at equivalent dilutions (data not shown).

Excessive osteoclast activity in non-union bone allografts

To gain better insight into the histological detail of bone allografts we performed histomorphometry of the host-graft bone interface. Several histomorphometric parameters of bone turnover were used to compare the cellular activity between union and non-union grafts. Reflecting our histological assessment, morphometric analysis revealed that mean values of OS/BS and QS/BS were significantly different for union and non-union allografts. Allografts in which union had occurred typically showed a higher proportion of osteoid covered surface, than did allografts with non-union (Fig. 5A,

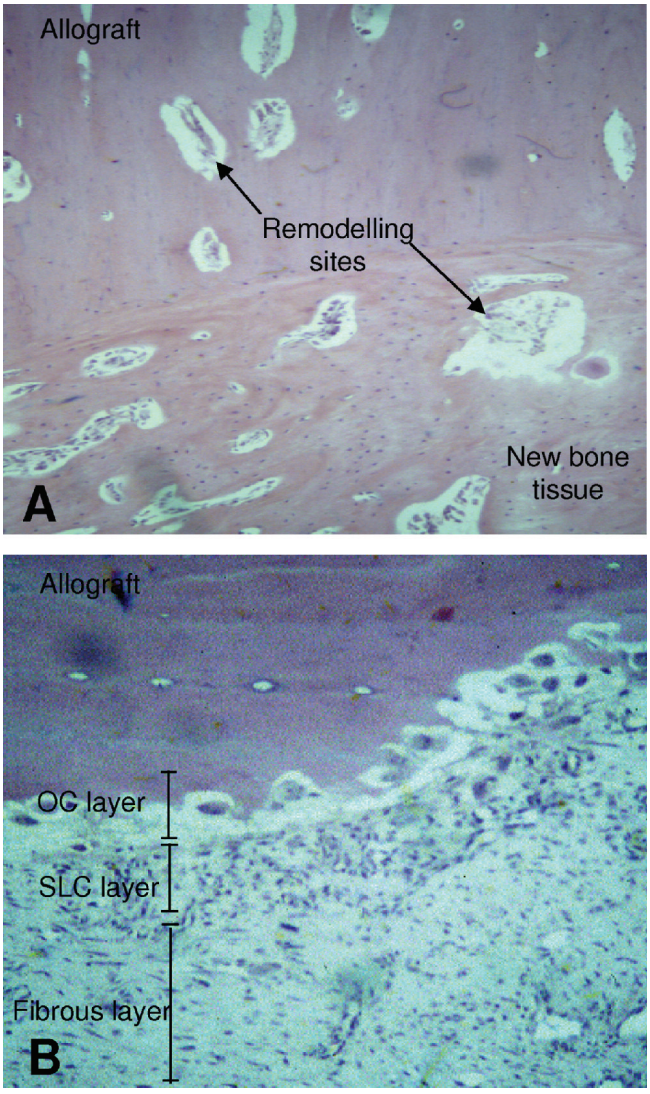

Fig. 2. Histological features of union and non-union femoral intercalary allografts. **A.** Union of host-bone allograft junction. Bone formation is observed on the surface of bone allograft. Multiple remodelling sites are also observed within cannels of the acellular allograft bone (arrows). x 200. **B.** Non-union of host-bone allograft junction. The allograft surface is highly eroded, with multiple resorption pits lined by multinucleated osteoclasts (OC). A densely cellular layer underlies the osteoclastic layer, predominantly comprised of large spindle-shaped mononuclear, synovial-like cells (SLC). The outer fibrous layer features vascular channels and flatter fibroblastic cells scattered throughout fibrous tissue x 200

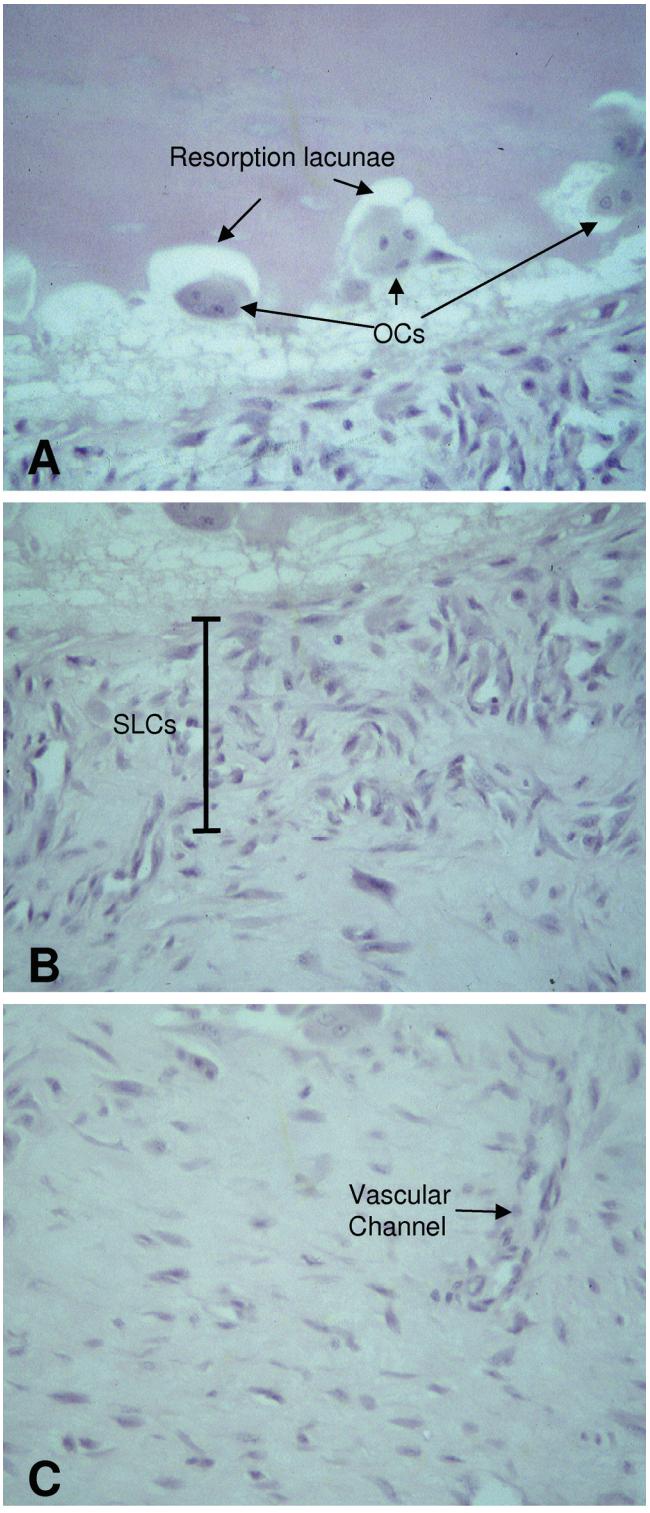

Fig. 3. Characteristic cell layers of typical non-union femoral intercalary allografts. **A.** Osteoclastic cell layer. Multiple OCs can be observed residing in resorptive lacuna. Note polygonal cells with synovial-like appearance lie directly adjacent this layer. **B.** Synovial-like cell (SLC) layer. **C.** Fibroblastic cell layer which is composed vascularised fibrous tissue. Arrow depicts vascular channel. x 400

$F_{1,27}$ = 19.461, P value < 0.001). Conversely, allografts with non-union displayed a significantly increased quiescent surface, compared to those in which union had occurred (Fig. 5B, F 1,27 = 4.692, P value = 0.04). Furthermore, in non-union allografts, the percentage of surface covered by resorption lacuna (ES / BS) (Fig. 5C, $F_{1.27}$ = 5.418, P value = 0.029), and that was in direct contact with OCs (OC.S / BS) (Fig. 5D, $F_{1,27}$ = 15.693, P value = 0.001), was significantly greater than in those in which union had occurred. Non–union allografts also displayed significantly increased numbers of OCs per mm of graft surface (N.Oc/BS) (Fig. 5E, $F_{1,27}$ = 6.279, P value = 0.019). However, the number of OCs in proportion to eroded surface (N.Oc/E.Pm) did not

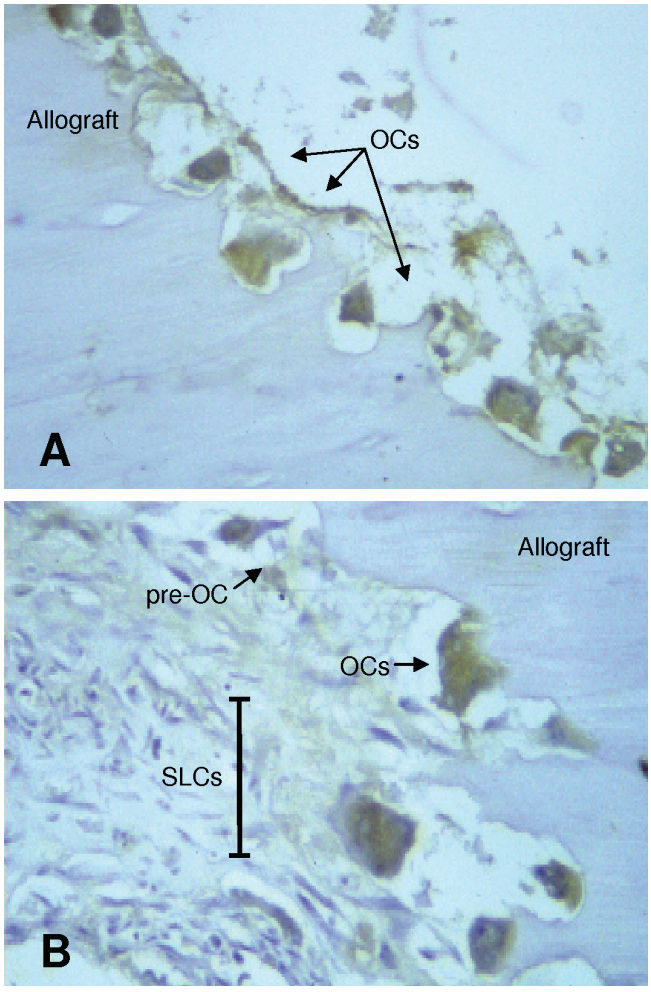

Fig. 4. Immunohistochemical detection of calcitonin receptors in bone-lining multinuclear cells. **A.** Multinuclear osteoclastic cells are positive for calcitonin receptor (arrows). x 400. **B.** Synovial like cells (SLCs) show little immunoreactivity to calcitonin receptor antibodies although scattered round shaped mononuclear cells and pre-osteoclastic cells (pre-OC) within the SLC layer exhibit moderate to weak immunoreactivity to calcitonin receptor, indicating the existence of mature OC precursor cells within the layer. x 400

change significantly between allografts with union and those with non–union (Fig. 5F, $F_{1,27} = 1.228$, P value = 0.279). Together, these data indicate that non-union allografts exhibit enhanced osteoclastogenesis.

Gene expression of RANKL in synovial-like cells

Excessive OC activity and/or numbers is a hallmark of a number of osteolytic diseases including osteoarthritis, peri-implant loosening, tumor-mediated bone loss and periodontitis (Helfrich, 2003). In each case, elevated OC activation has been shown to correlate with an increase in the expression levels of receptor activator for nuclear factor kappa B (NF-κB) ligand (RANKL), a transmembrane protein known to directly stimulate OC formation (Huang et al., 2000; Haynes et al., 2001a,b; Liu et al., 2003). Thus, we hypothesized that the enhanced osteoclastogenesis observed at the non-union sites might similarly reflect enhanced production of RANKL by surrounding cell types. To explore this possibility we performed *in situ* hybridisation on bone allograft sections using a DIG-labelled RANKL probe. As shown in Fig. 6, RANKL mRNA expression was indeed detectable in both non-union (Fig. 6A) and union (Fig. 6B) allografts. In non-union cases, RANKL mRNA was abundantly expressed throughout the synovial-like cell layer (Fig. 6A). What is more, RANKL expression was highly specific and invariably intense in the cytoplasm of synovial-like cells, with little to no positive signal detectable in the osteoclastic or fibrous layers. By comparison, RANKL expression was moderate to weak in all union cases examined, being largely confined to osteoblasts and stromal cells lining the host-allograft bone interface and residing in areas of internal remodelling (Fig. 6B). RNAse treatment prior to hybridisation resulted in loss

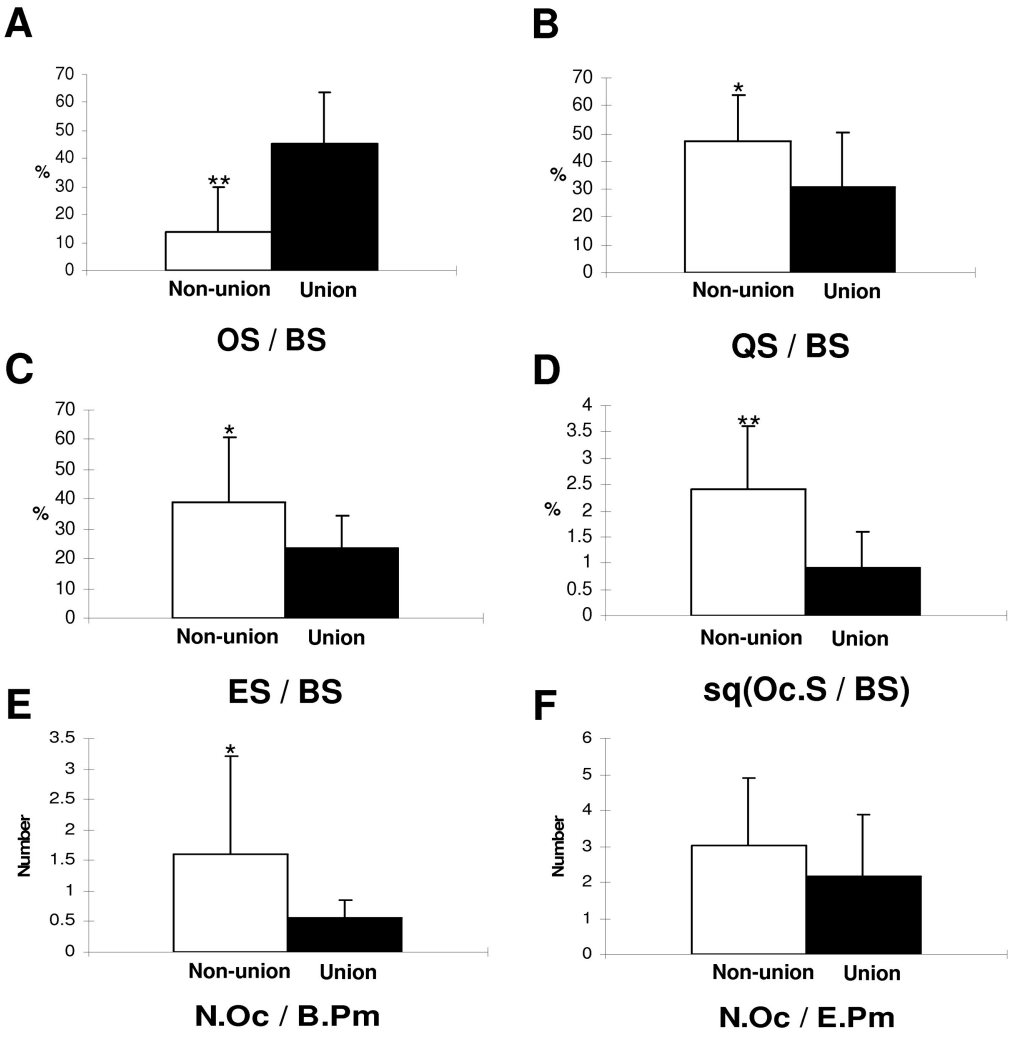

Fig. 5. Histomorphometric assessment of union and non-union bone allografts. Measurements of osteoid surface per bone surface (OS/BS; **A**) quiescent surface per bone surface (QS/BS; **B**) eroded surface per bone surface (ES/BS; **C.** OC surface per bone surface squared sq(Oc.S/BS; **D**) number of OCs per mm of graft bone surface (N.Oc/B.Pm; **E**) and number of OCs per mm of eroded surface (N.Oc/ E.Pm; **F**) on union and non-union bone allograft. See Material and Methods. Data presented as Means + SD. (P-value= *<0.05, **<0.001).

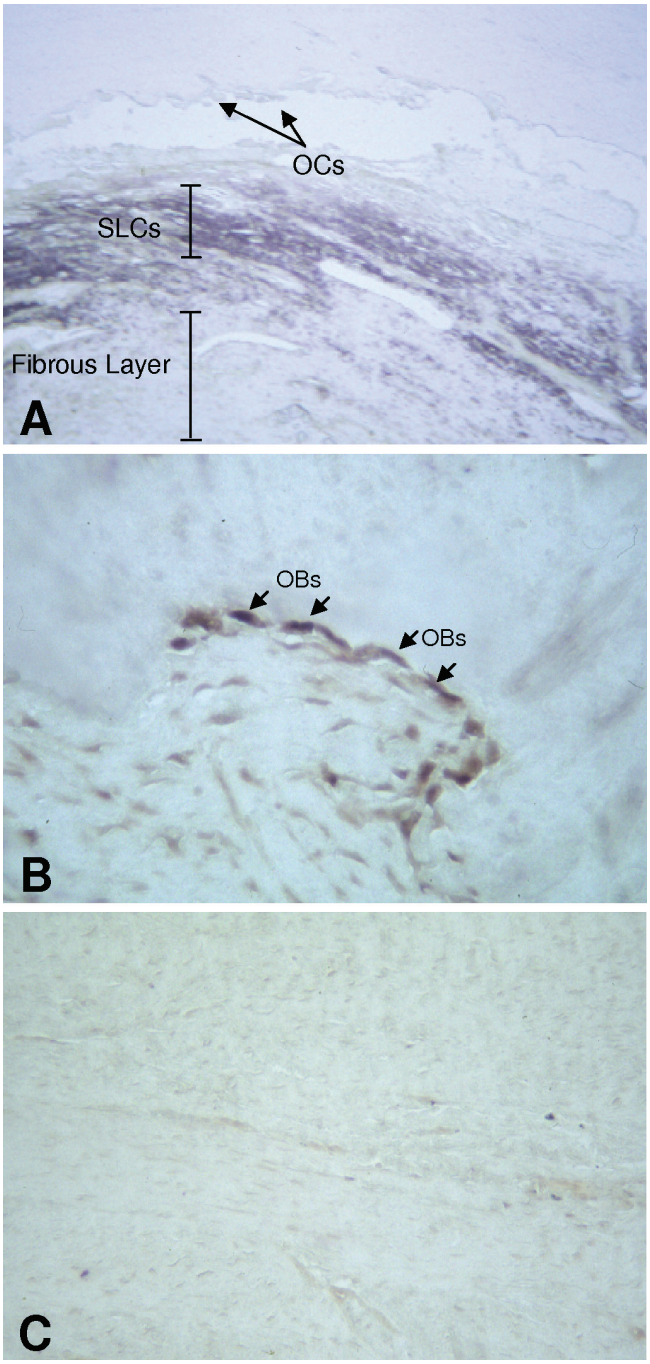

Fig. 6. *In situ* detection of RANKL expression in bone allografts. **A.** A low magnification (x 100) of a non-union graft section. Note, an abundance of RANKL expression (brown reaction product) is evident in the synovial-like cell (SLCs) layer whereas little to no signal is detectable in osteoclastic and fibroblastic layers. **B.** In union of bone allografts, RANKL mRNA expression is predominantly detectable in the cytoplasm of osteoblasts located on surface of allograft bone. x 200. **C.** *In situ* hybridisation following RNAse treatment shows only background activity as served as a negative control. x 100

of signals in all cells, indicating the specificity of the probe for its target mRNA sequence and thus served as a negative control (Fig. 6C). In all, these findings indicate that synovial-like cells within non-union allografts abundantly express RANKL mRNA suggesting that, like in other OC-mediated disorders, the increase in OC activity is related to an enhancement or up-regulation of RANKL in these grafts.

Discussion

Despite the union of bone allografts with host bone being widely documented there is comparatively little information regarding the histopathology of instances of non-union cases. The results of the present histological investigation provide new insights into the cellular mechanisms underlying the repair sequences in both union and non-union intercalary bone allografts. Typically, all allografts with union displayed evidence of osteogenesis and osteoconduction. This histological appearance is in line with previously reported allograft repair sequences in which allografts characteristically displayed inflammation, union, vascularisation and creeping substitution, before finally undergoing host-graft bone remodeling (Burchardt, 1983; Aro and Aho, 1993; Garbuz et al., 1998). In contrast to these reactions, allografts which failed to unite displayed several unique histological characteristics. Among these, excessive osteoclastic bone resorption was prevalent suggesting that increased OC activity may contribute directly to bone allograft failure. This observation is consistent with previous findings that a higher rate of bone resorption than bone formation increases the risk of bone allograft failure (Li et al., 1991; Delloye et al., 1992; Thompson et al., 2000; Zabka et al., 2001).

In all non-union allografts examined there was a large expanse of eroded allograft surface which was predominately lined by multinucleated OCs within resorption lacunae. Underling this layer was a belt of spindle-shaped synovial-like cells, and circumscribing this, a layer largely composed of fibrous tissue. Furthermore, very little bone formation or osteoblastic activity was observed at the junction regions of non-union allografts. These histological changes are analogous to the tissue observed surrounding mobilized prostheses (Bonucci, 2003). Reflecting these changes, histomorphometric studies revealed that non-union allografts possessed increased numbers of OCs in proportion to the graft surface, and had a significantly larger total eroded perimeter. On the other hand, the number of OCs per eroded perimeter (N.Oc/E.Pm) was found to be approximately equal for union and non-union allografts and both had a similar amount of eroded surface relative to the number of OCs surrounding the graft. Thus it appears that non-union allografts had a larger osteoclastic presence and a larger proportion of eroded surface than union cases, suggesting that excessive bone resorption is due to the induction of osteoclastogenesis. In short, these studies demonstrate

that allograft non-union correlates with increased OC numbers and resorptive activity together with lack of osteogenesis at the host-allograft junction.

It has been previously suggested that a higher rate of bone resorption than bone formation in allograft failure may be due to the incompatibilities of major histocompatibility complex (Enneking and Mindell, 1991; Griffiths et al., 1995). The latter leads to the recruitment of reactive foreign body multinucleate giant cells and subsequent inflammatory changes (Enneking and Mindell, 1991). In the present study however, we did not observe any obvious evidence of an inflammatory response nor the presence of foreign body reactive multinuclear giant cells in non-union allografts. Although we did identify a number of multinucleated cells at the host-allograft bone junction in non-union cases, these cells were identified as OCs and not reactive foreign body giant cells as evidenced by their positivity to calcitonin receptor immunohistochemistry. Thus, while there is no doubt immune responses plays an important role in the repair of bone allografts (Friedlander, 1987), the exact relationship between the induction of excessive bone resorption in non-union allografts and immune-rejection remains somewhat unclear. Nonetheless, given the striking similarities which exist between the histopathology of non-union cases with that observed in peri-prosthetic loosening, we speculate that the enhanced bone resorption in allograft non-union reflects increased recruitment and differentiation of OCs. It is possible that the degree of osteoclastogenesis is directly dependent on the level(s) of cytokines, including IL-6 and TNF-α as observed in aseptic loosening (Greenfield et al., 2002; McEvoy et al., 2002). Indeed, our results suggest that the underlying synovial-like cells may contribute, at least in part, to the excessive osteoclastic bone resorption through the production of RANKL. The latter of which is considered to be the "master cytokine" necessary for the formation and activation of OCs. RANKL is known to bind with high affinity to it's cognate receptor RANK (receptor activator for NF-κB), which is present on the surface of OC precursors, and directly stimulates OC precursors to differentiate into OCs (Lacey et al., 1998; Yasuda et al., 1998; Xu et al., 2000).

While our *in situ* findings unequivocally demonstrate that synovial-like cells express a preponderance of RANKL, the precise nature and ontogeny of these cells requires further investigation. Thus far, our attempts to better characterize these cells using established osteoblastic and fibroblastic markers such as (osteoprotegerin, osteopontin, alkaline phosphates, type I collagen, Runx-2) have been precluded largely due to a lack of appropriate reagents to sheep. Nonetheless, based on their morphology and the fact that they express RANKL, it is probable that these cells are synovial fibroblasts and comparable to those observed previously in rheumatoid arthritis sufferers (Takayanagi et al., 1997). It is tempting to speculate that, in non-union cases, these synovial fibroblasts may induce macrophages to undergo osteoclastogenesis, or, alternatively, target other pre-osteoclastic populations. In support of this position, calcitonin receptors were detected in small, round-shaped mononuclear pre-osteoclastic cells within the synovial-like cell layer, suggesting the existence of late-stage differentiated OC precursor cells in this cell layer.

Taken as a whole, the results of this histological study have provided evidence to suggest that excessive osteoclastic bone resorption attributes to the non-union and failure of bone allografts. Moreover, these studies hint that the over-production of RANKL by cells resembling synovial fibroblasts may be responsible for the enhanced generation and activation of OCs in non-union allografts. It therefore may be possible that enhancement of bone allograft union can be achieved by (1) the administration of anti-bone resorption agents such as bisphosphonates; or (2) through the use of inhibitory peptides against RANK (eg RANK:Fc) to block OC differentiation. In fact, the later approach has been recently employed to prevent wear debris-induced osteolysis in mice (Childs et al., 2002). Whether such treatments can be translated to allograft non-union, together with the delineation of the synovial-like cell ontogeny, will be the focus of future studies.

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