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Observations on bone formation and remodelling in advanced atherosclerotic lesions of human carotid arteries

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Abstract Immunolocalisation and histochemical techniques were used to examine mineralised bone deposits within late stage atherosclerotic plaques of human carotid arteries. These specimens showed characteristic features of osteogenesis. Large calcifications were often observed in close association with or integrated within mineralised bone. Smooth muscle cells (α-actin positive) were often located around osteoid-like matrix, together with focal accumulations of macrophages (CD68 and HAM56 positive). Local accumulations of mast cells (tryptase positive) were consistently observed in close association with the bone. Multinucleated giant cells in close apposition with mineralised bone demonstrated typical osteoclastic morphology, and were positively stained for acid phosphatase and the macrophage marker CD68. Thus, all the normal features of bone formation and resorption were observed in this microcosm of osteogenesis within atherosclerotic plaque; the term 'osteosome' seems appropriate for the structure. These osteosomes have numerous advantages for experimental studies of the various osteogenic factors responsible for bone metabolism, especially following short-term tissue culture. This ex vivo technique was used to demonstrate the distribution and the multiple cellular sources of bone morphogenetic protein 6.

Key words Atherosclerosis · Osteogenesis · Mineralisation · BMP-6

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Introduction

Heterotopic bone formation is not uncommon and may occur as a consequence of tumours, infections, generalised or focal disease, or various forms of trauma [3, 26]. Dystrophic calcification is not unusual in late stage atherosclerosis [2, 25, 29]; indeed, calcification is a prominent feature of atherosclerotic lesions. It is evident even from stage III of plaque development [19] and is thought to contribute to the biomechanical changes of the artery wall and the clinical complications associated with cardiovascular disease. In a minority of late stage atherosclerotic lesions the mineral has the histological appearance of fully formed bone with trabeculations and cellular inclusions resembling bone marrow [6]. Such observations on autopsy aortic material led Virchow, in 1863, to describe arterial calcification as 'ossification' [31].

Recent evidence suggests that calcification of atherosclerotic arteries is an organised, regulated process, rather than a passive phenomenon of ageing [6, 7, 32]. Several factors related to bone and mineral formation have been demonstrated within atherosclerotic plaques over recent years; these include osteocalcin, osteopontin, osteonectin and bone morphogenetic proteins 2a and 6 [4, 10, 27, 32]. The presence of these factors indicates the potential of arterial wall cells to promote calcification and osteogenesis in certain conditions, but the cellular origin of each factor remains uncertain. Similarly, it is not clear whether calcification is related directly to bone formation, or how mineralisation is induced in specific microenvironmental sites of the artery wall.

During our histological studies on calcification in atherosclerotic arteries [19] we recognised mineralised bone deposits in some of the late stage specimens. This provided an opportunity to examine the site of osteogenesis with regard to the distributions of specific cell types and the relationship with associated calcification. The cellular components observed at these sites of mineralisation support the concept that heterotopic bone formation in atherosclerotic plaque manifests all the cellular features commonly associated with osteogenic remodel-

ling in skeletal bone. We have subjected mineralised artery walls to short-term organ culture to examine the expression and distribution of osteogenic factors such as the bone morphogenetic proteins (BMPs), growth and differentiation factors that are part of the TGF-β superfamily and stimulate endochondral ossification processes by mesenchymal cells [14, 15]. Use of this organ culture technique has provided information on the cellular origin of one of these factors: bone morphogenetic protein 6 (BMP-6), a growth factor that is reported to have a central role in mesenchymal differentiation and bone formation [15].

Materials and methods

Samples of atherosclerotic carotid arteries were collected from 101 patients undergoing surgery in the University Department of Surgery, Withington Hospital, Manchester, UK. The material consisted of endarterectomy specimens (n=90) and full-thickness resections (n=11) of common carotid artery, frequently with bifurcation and an adjacent portion of the internal branch.

Immediately after resection the specimens were placed in Dulbecco's modified Eagles' medium (DMEM); within minutes they were fixed in Carnoy's fixative for 4–6 h and then processed to paraffin blocks. Specimens were sectioned at 3 µm, or 5 µm in heavily calcified material, placed on glass slides treated with poly-L-lysine (Sigma), dewaxed in Histoclear, rehydrated in a graded series of ethanol, and stained with haematoxylin and eosin. Each specimen was assessed for the stage of plaque development according to the classification described by Stary et al. [29] and subsequently examined for calcification using either haematoxylin or the von Kossa staining technique [1], followed by counterstaining with Safranin O. Specimens which showed signs of heavy calcification or mineralised bone were subsequently examined for the distribution of mast cells, using immunolocalisation with the monoclonal antibody to mast cell tryptase (Chemicon International, London, UK), macrophages, using immunolocalisation with the monoclonal antibodies to CD68 (KP1 and PG-M1) and HAM56 (Dako, Glostrup, Denmark), smooth muscle cells (SMC), using immunolocalisation with antibody to smooth muscle actin clone 1A4 (Dako), bone morphogenetic protein 6 (BMP-6), using monoclonal antibody (Novocastra Laboratories Ltd, Newcastle, UK), and multinucleated osteoclast-like cells stained for tartrate-resistant acid phosphatase (Burnstone method modified by Barka [1]).

Secondary antibodies of biotinylated rabbit anti-mouse IgG and goat anti-mouse IgG were purchased from Dako.

Tissue sections were incubated with 10% (v/v) non-immune rabbit serum for 30 min and then treated with one of the primary monoclonal antibodies for 2 h at room temperature. For the monoclonal actin antibody tissue sections were pretreated with trypsin (from Dako, used according to specification). After washing in TBS (3×10 min) the sections were incubated with secondary biotinylated rabbit anti-mouse antibody for 45 min. After further washing in TBS, StreptABComplex alkaline phosphatase (AP) conjugate (Dako) was applied for 45 min, after which they were washed again with TBS, and alkaline phosphatase was developed using New Fuchsin. Tissue sections were counterstained with Mayer's haematoxylin, mounted in Histomount (Mensura Technology, Wigan, UK), examined with a Zeiss Photomicroscope III, and photographed using Ektachrome 160 tungsten film.

For dual immunolocalisation tissue sections were treated for endogenous peroxidase (using 1% hydrogen peroxide in absolute methanol for 15 min) and incubated with 10% non-immune rabbit serum and then with primary antibodies to the macrophage markers. Bound antibody was visualised using a biotinylated rabbit anti-mouse IgG and a streptavidin biotin immunoperoxidase procedure with 3,3'-diaminobenzidine as a chromogen (brown stain). Sections were subsequently treated with 0.1 mol/l glycine-HCl (pH 2.2) for 2 h to remove all trace of the first primary antibody, to avoid cross-immunolabelling [18]. Slides were washed extensively in TBS, incubated with 10% non-immune goat serum, and then with mouse anti-human tryptase followed by a goat anti-mouse alkaline phosphatase-conjugated IgG. Alkaline phosphatase was developed using New Fuchsin (red stain).

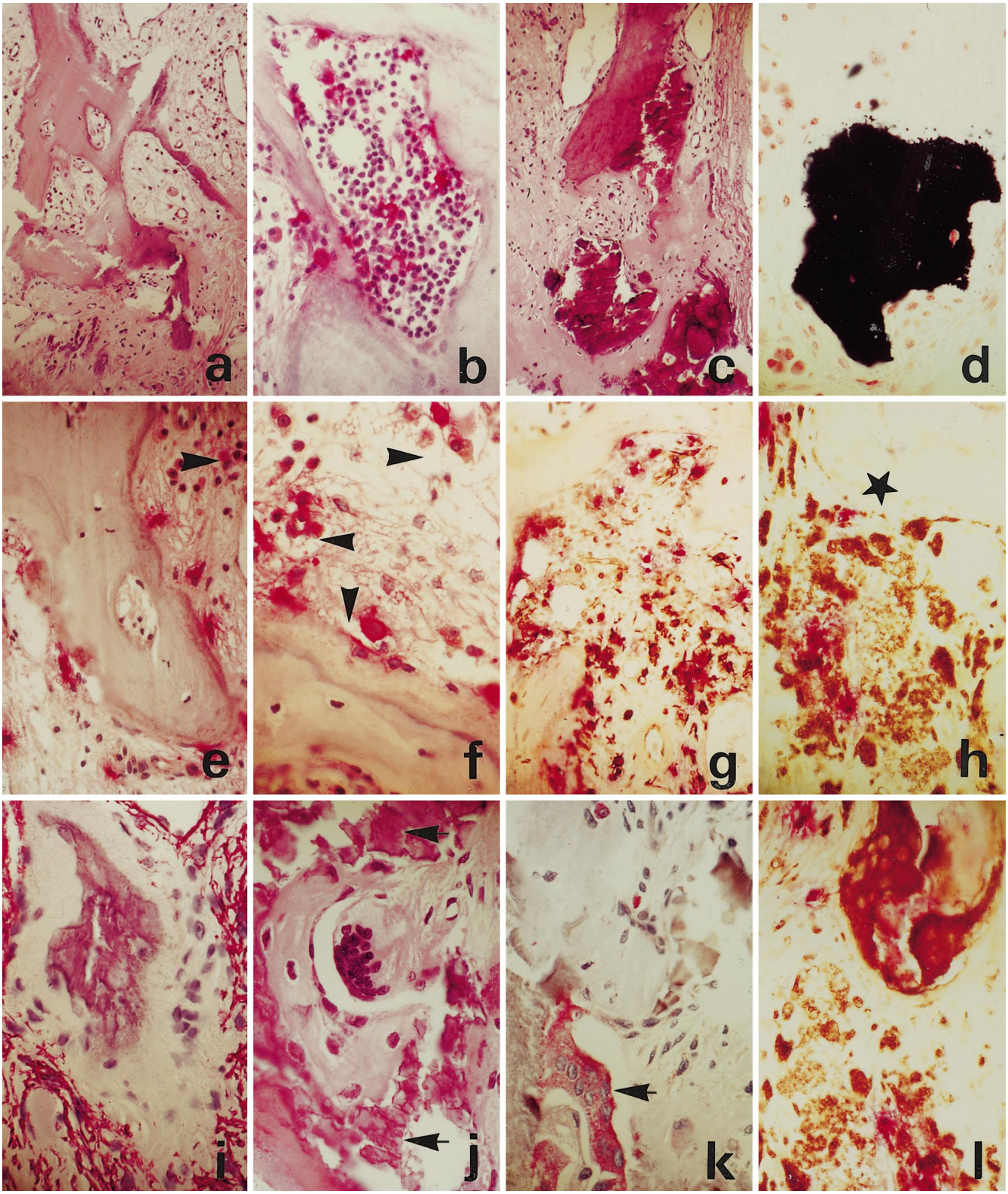
Late stage atherosclerotic plaques (n=15) with heavy mineralisation, as judged by 'hardened' artery walls, were selected for culture studies. Under aseptic conditions the tissue was cut into explants of approx. 4 mm^3 and cultured in Dulbecco's modified Eagles' medium (DMEM) in a humidified CO_2 incubator (5% CO_2 in air) at 37°C. After 24 h and 48 h in culture the explants were processed for histology as described above for the immunolocalisation of BMP-6, macrophages and SMC.

Results

Mineralised bone was initially identified in late stage atherosclerotic plaques using H&E-stained sections of undecalcified material (Fig. 1a). Von Kossa staining was also used to demonstrate and confirm the calcification and bone structures within each specimen (Fig. 1d). Three carotid specimens in particular showed extensive microfocal distributions of mineralised bone, occupying almost half of the dorsal circumference of the artery wall, but in no more than about a 4 mm segment of each plaque. Five other specimens contained foci of osteoid-like matrix in calcified plaque tissue. These specimens presented similar histological features with regard to the distribution of specific cell types. Bone trabeculae were mostly thin and irregular with relatively few osteocytes. Marrow-like inclusions in one specimen contained lymphocytes, tryptase-positive cells and thin-walled vasculature (Fig. 1b), together with numerous CD68 positive monocytes (not shown). Relatively large calcified deposits were observed in close association with, or integrated within, the bone matrix (Fig. 1c).

Peripheral accumulations of mast cells, identified by tryptase immunostaining, were a common feature

Fig. 1a–l Histological observations of heterotopic bone formation in atherosclerotic plaque of human carotid arteries. **a** Low-power micrograph of arterial intima, showing mineralised bone trabeculae in vascular stroma. Haematoxylin and eosin, ×100 **b** Micrograph of marrow-like inclusion within mineralised phase, showing lymphocytes, mast cells (tryptase, *red*) monocytes and thin-walled blood vessels. Tryptase immunostaining, counterstaining with haematoxylin, ×270 **c** Low-power micrograph showing calcifications (dark blue-red stain), associated with and integrated within, mineralised bone. Haematoxylin and eosin, ×100 **d** Von Kossa staining (*black*) shows heavily calcified bone. Safranin O counterstain shows osteocytes and surrounding cells. ×270 **e** Microphotograph showing close association of mast cells (tryptase, *red*) with the periphery of the bone, together with a small group of tryptase-positive mononuclear cells (*arrow*). Tryptase immunostaining, counterstaining with haematoxylin, ×270 **f** High-power micrograph, showing a very close association of mast cells (tryptase, *red*) with osteoid-like covering of bone. Note extracellular tryptase, suggestive of mast cell activation, and local oedema and stromal disruption (*arrows*). Note also the osteocytes typical of mineralised bone. Staining as in (**c**). ×270 **g** Low-power micrograph showing intermixed populations of mast cells (tryptase, *red*) and macro- ▶



phages (HAM56, *brown*) at periphery of bone. ×100 **h** High-power micrograph showing a mixture of macrophages (HAM56, *brown*) together with a few mast cells and extracellular tryptase (*red*) in close association with mineralised bone (*asterisk*). ×100 **i** Smooth muscle cells immunostained with α-actin (*red*) surrounding osteoid-like matrix with central calcification. Haematoxylin counterstain, ×270 **j** Giant cell in apposition with an arcuate bone trabeculum. Note associated calcifications (*arrows*). Haematoxylin counterstain, ×270 **k** Multinucleated giant cells stained for acid phosphatase (red, *arrow*) typical of osteoclasts. Note close contact on left side with mineralised bone. Haematoxylin counterstain, ×270 **l** Multinucleated giant cells and macrophages stained for CD68 (*brown*) intermixed with degranulated mast cells (extracellular tryptase, *red*) associated with osteoid-like matrix. ×270

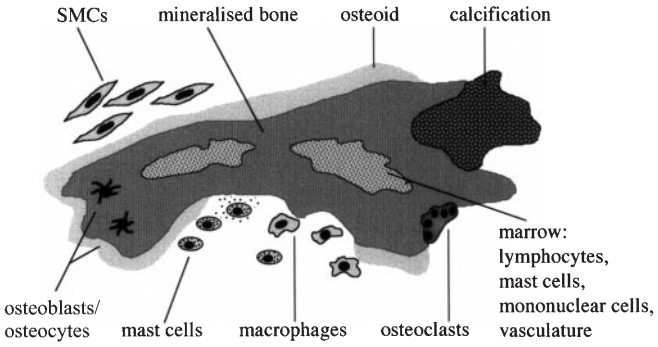


Fig. 2 Summary diagram of an atherosclerotic 'osteosome' to illustrate the microenvironmental nature of specific cell distributions

around most of the bone deposits (Fig. 1e), their numbers being far greater than in adjacent stromal tissue. This very close association of mast cells with mineralised bone often corresponded with microfocal sites suggestive of matrix remodelling. For example, Fig. 1f shows evidence of mast cell activation, as judged by extracellular tryptase staining, associated with an area of local oedema and stromal matrix disruption immediately peripheral to an osteoid-like covering of mineralised bone. Mast cells were also observed intermixed with macrophages and numerous blood vessels (Fig. 1g, h), again in microenvironmental sites around the bone deposits. Macrophages, stained with the HAM56 marker, showed a variable distribution around mineralised bone, but they were typically a major cellular component of more remote inflammatory and lipid-rich sites of the plaque. Similarly, stromal fibroblasts, presumably transformed SMC, showed a variable distribution; they were often observed around osteoid-like matrix (Fig. 1i), but were less prominent around fully developed bone enveloped in a relatively acellular fibrous matrix. However, whereas the locations containing SMC or a fibrous overlay demonstrated a well-ordered extracellular matrix, those containing macrophages and mast cells were commonly associated with a disorganised matrix suggestive of oedematous and degradative changes (Fig. 1g, h).

Multinucleated giant cells were observed in close apposition to the mineralised phase of the bone deposits (Fig. 1j–l). Such cells were not seen in the stroma surrounding the mineralised structures, although giant cells may represent a component of the atheromatous lesions of plaque. The giant cells associated with mineralised substrates not only demonstrated an osteoclastic morphology, but also stained positively for acid phosphatase (Fig. 1k) and the CD68 marker (Fig. 1l), which is indicative of a macrophage lineage. These observations of osteoclastic cells, presumably reflecting resorptive activity, together with the formation of osteoid-like matrix and mineralisation, suggest that bone remodelling processes are manifested within such mineralised atherosclerotic artery walls.

Figure 2 is a schematic summarising the observations of specific cell types associated with these mineralised structures; it also highlights the microenvironmental nature of the potential synthetic and resorptive processes responsible for osteogenesis and bone remodelling.

Bone morphogenetic proteins (BMPs) are recognised as important osteogenic factors, and BMP-6 is reported to induce bone formation from mesenchymal cells at extraskeletal ectopic sites [15, 16]. BMP-6 was demonstrated in freshly fixed mineralised plaques around several of the mineralised bone structures, either at microfocal sites as depicted in Fig 3a, or around the peripheral margins of the bone structures (Fig. 3b). Occasionally the osteocytes within the bone matrix were positive for BMP-6. However, histological studies of specimens fixed shortly after excision did not give a clear indication of the cellular source(s) of this factor. This problem was resolved to some extent by the use of short-term explant cultures of heavily calcified plaque specimens. Subsequent immunolocalisation studies of these mineralised artery wall explants showed that SMC were a major source of BMP-6 (Fig. 3c, c') and that to a lesser extent giant cells and a small proportion of macrophages could also elaborate this protein (Fig. 3d, d').

Discussion

Earlier observations on the calcification and 'hardening' of arteries have conventionally been performed on aortas and coronary arteries [29]. This study has examined the specific cellular associations with heterotopic bone formation in atherosclerotic carotid arteries using plaque specimens derived from endarterectomy procedures. However, it is probable that our findings are similar to the bone formation observed in other types of atherosclerotic artery. The major conclusion is that bone formation in the intima of atherosclerotic plaque demonstrates many features that are typical of skeletal osteogenesis, including osteoid, mineralised trabeculae, osteocytes, marrow-like inclusions with lymphoid and monocytic cells, multinucleated osteoclasts, and various inflammatory cells such as mast cells and macrophages. Moreover, the microenvironmental nature of the specific cell distributions demonstrates that both anabolic and catabolic processes are probably operating in most of these heterotopic macro- or microscopic bone structures.

The presence of mast cells and macrophages in microenvironments at the interface with osteoid-like or mineral surfaces was a common observation. Both cell types may express numerous proinflammatory and reparative mediators, including cytokines, prostanoids, growth factors and proteinases [11, 12, 18]. Mast cells have previously been reported on the endosteal bone surface of rats showing secondary parathyroidism and rickets [30] and in bone marrow, where mast cells are especially numerous in osteoporosis [8, 9], observations suggesting that mast cells might increase bone loss at trabecular and

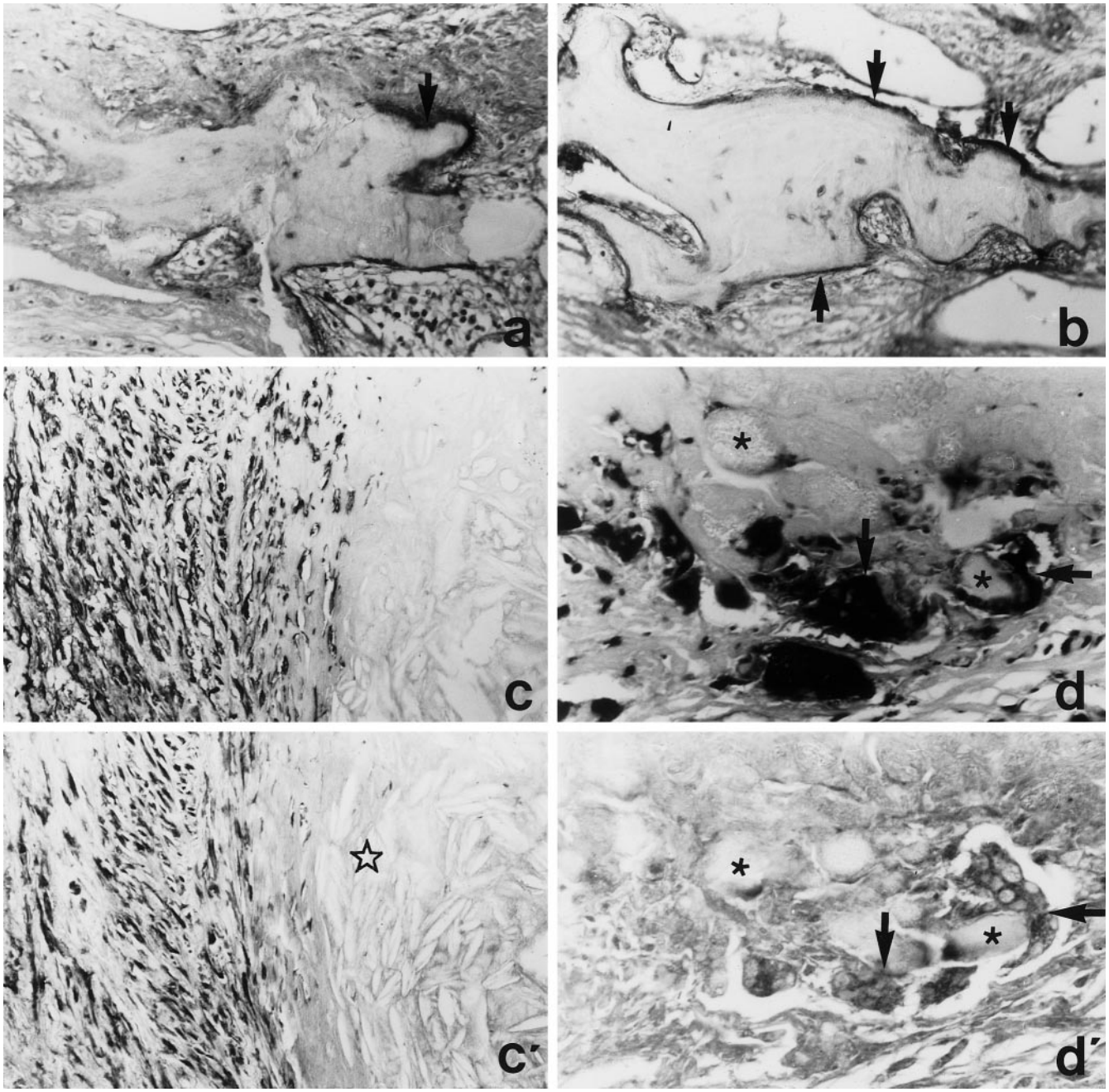


Fig. 3a–d Distribution of bone morphogenetic protein 6 (BMP-6) in late stage, mineralised atherosclerotic plaques of carotid arteries and identification of its cellular source. **a, b** Immunolocalization of BMP-6 **a** in microfocal (*arrow*) and **b** at the peripheral margins of (*arrows*) mineralised bone structures. Note also the extracellular staining and some osteocytes positive for BMP-6. No counterstain, ×130 **c, c'** Deep margin of atherosclerotic plaque after 24-h ex vivo tissue culture immunostained for **c** smooth muscle cells (SMC) and **c'** BMP-6. Note the co-distribution of BMP-6 with most of the SMCs, and its absence from the lipid core (*asterisk*). No counterstain, ×130 **d, d'** Calcified atherosclerotic plaque after 24-h ex vivo tissue culture showing **d** CD68-stained macrophages and giant cells (*arrows*) and **d'** the co-distribution of weakly stained BMP-6 in the giant cells (*arrows*) and a few macrophages close to calcium deposits in a consecutive section. Calcifications indicated with (*asterisks*). No counterstain, giant cells confirmed by H&E on adjacent section, ×300

endosteal surfaces [22]. Mast cell products such as heparin, TNF-α, IL-1 and PGD_2 are reputed to influence bone catabolism [22], whereas histamine, prostaglandins and TGFβ have been implicated in peritrabecular and skin fibrosis [17, 20, 22], observations that support the notion of a multifactorial role for the mast cell in bone remodelling processes.

New bone formation requires an extracellular matrix composed of various collagenous and non-collagenous proteins, proteoglycans, osteogenic factors and, ultimately, a mineral phase based on hydroxyapatite [24]. Most osteogenic factors are not normally expressed in artery walls, and the microenvironmental conditions within atherosclerotic plaque that give rise to calcification and osteogenesis are poorly understood. The forma-

tion of bone by osteoblasts, in this study probably equivalent to transformed mesenchymal cells such as intimal SMC, and the remodelling brought about by osteoclasts, is a closely regulated system. Reddi [24] has used the term “osteosome” to describe the smallest unit of bone formation, and this seems an appropriate description of the microscopic structures of bone examined here (see Fig. 2).

Calcification of atherosclerotic plaque is thought to be an organised and regulated process with many similarities to the mechanisms associated with bone formation [5–7, 10]. Thus it was not surprising to see relatively large calcifications clearly associated with, or even integrated within, the bone fragments examined in this study. Although peripheral, osteoid-like matrix enveloped some portions of mineralised bone, its composition relative to chondroid/cartilage components remains uncertain. Both calcification and osteogenic processes are dependent upon the production within plaques of several factors such as osteocalcin, osteopontin, osteonectin and BMPs [4, 10, 13, 27, 32]. Osteopontin and osteonectin have been reported in atherosclerotic plaques and implicated in the mineralisation process [10, 16, 28], and platelet-derived growth factor accelerates fracture healing and bone repair as an osteoblast mitogen [23]. BMPs induce the in vivo transformation of extraskeletal mesenchymal tissue into bone or cartilage [4, 14, 15], and in a recent study BMP-6 immunoreactivity was demonstrated in SMC of intimal atherosclerotic plaques and normal cerebral blood vessels [27]. The present study has demonstrated BMP-6 at peripheral locations of intimal bone formation; however, its cellular distributions were more clearly defined in 24-h explants of mineralised plaque, where its production was identified in SMC, osteocytes, giant cells and a minority of the macrophages. Thus, these short-term culture experiments have produced more convincing evidence that several cell types can elaborate BMP-6, observations that were not so apparent in freshly fixed specimens. We believe that this short-term ex vivo technique provides numerous advantages for the further study of factors involved in atherosclerotic calcification, osteogenesis and bone resorption; especially since the heterotopic osteosomes described appear to provide a unique microscopic model of normal bone remodelling processes.

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