European Journal of Orthodontics 20 (1998) 357–367

Enhanced angiogenesis induced by diffusible angiogenic growth factors released from human dental pulp explants of orthodontically moved teeth

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SUMMARY The aim of this study was to determine if diffusible angiogenic growth factors were released in human dental pulp during orthodontic tooth movement. These factors, if diffusible, could induce angiogenesis in other tissues, and may then be isolated and identified. The pulps from 14 premolar teeth treated with straight wire fixed orthodontic appliances for 2 weeks were compared with those of 14 untreated control premolar teeth from the same subjects. Following tooth extraction and sectioning, 1-mm horizontal sections of pulp tissue were embedded in collagen with 1-mm sections of rat aorta and co-cultured in growth media for up to 4 weeks. Sections of rat aorta alone were also cultured. Angiogenic changes in the form of microvessel growth were observed by light microscopy. Microvessel identification was confirmed by electron microscopy and by immunohistochemistry using staining for factor VIII-related antigen marker for endothelial cells.

When compared at days 5, 10 and 14 of co-culture, the number of microvessels was significantly greater in the pulps from orthodontically moved teeth than in those from the control teeth. The number of rat aorta microvessels was also significantly greater when co-cultured with pulp from orthodontically moved teeth than with pulp from control teeth and when compared with control cultures of rat aorta alone. There were no significant differences in microvessel numbers between the rat aorta co-cultured with pulp from control teeth and control cultures of rat aorta alone. These results indicate an increase in angiogenic growth factors in the pulp of orthodontically moved teeth, and the enhanced response of the rat aorta when co-cultured with this pulp shows that these factors appear to be diffusible.

Introduction

The role of angiogenic growth factors in orthodontic tooth movement requires investigation. Angiogenesis, the formation of new capillary structures, is initiated and regulated by a number of polypeptide growth factors (Folkman and Klagsbrun, 1987). The angiogenic response is a cumulative effect of positive and negative regulatory factors. Angiogenic growth factors have been identified in wound healing and hypoxic/ischaemic tissues in the body (Schultz and Grant, 1991), where they may be of blood borne origin or synthesized locally by vascular endothelial cells and released following cell injury. Changes in oxygen tension or factors released from lysing cells in ischaemic tissue may initiate angiogenesis.

Following orthodontic force application, mechanical damage to pulpal capillaries similar to that reported in the periodontium (Rygh *et al.*, 1986), circulatory disturbances and inflammatory changes (Mostafa *et al.*, 1991) and changes in oxygen levels in the pulp (Unsterseher *et al.*, 1987) may result in a similar pattern of events to that initiating angiogenesis in wound healing. During a lengthy course of fixed appliance orthodontic treatment, repeated force application could lead to repeated cycles of mechanical injury, inflammation and wound healing, and may therefore

involve repeated stimulation and release of angiogenic growth factors in the pulp.

The results of an initial investigation of the angiogenic response of the dental pulp to orthodontic force (Derringer *et al.*, 1996) support the hypothesis that there is an increase in angiogenic growth factors in the pulp during orthodontic tooth movement.

The aim of this study was to determine if the angiogenic growth factors released were diffusible and could therefore induce angiogenesis in the rat aorta when co-cultured with pulp from orthodontically moved teeth. If diffusible, these factors could induce angiogenesis in other tissues and may then be isolated and identified.

Materials and methods

Materials

All materials were purchased from Sigma Chemical Co. (Poole, UK) except where otherwise stated in the text.

Human dental pulp and rat aorta co-culture assay

The three-dimensional proliferative assay technique used in this study was based on that of Nicosia and Ottinetti (1990), and modified by Derringer *et al.* (1996).

The dental material consisted of 28 premolar teeth from orthodontic patients requiring extractions and fixed appliance treatment. Ethical approval was given by the Research Ethics Committee at King's College Hospital on condition that only patients who wished to participate with informed parental consent were included in this study. Patients were aged in the range of 11–14 years and the teeth used were dentally healthy (i.e. free of caries and restorations).

In each patient, orthodontic force (in the range of 0.5–1 N in a mesial and extrusive direction) was applied to two premolar teeth while the contralateral premolar teeth were used as untreated intra-patient controls. Fixed appliance (Andrews 0.022-inch, Forestadent, Milton Keynes, UK) straight wire orthodontic brackets were direct bonded (Concise, Dental Express, Kent, UK) to the upper and lower teeth from second premolar to second premolar, except for the two control premolar teeth. Orthodontic bands were cemented (Ketac, Baxter Dental, Watford, UK) onto all first permanent molar teeth. Test tooth position was carefully assessed and brackets bonded in a position so that the archwires placed gave the required force to the test teeth. After 2 weeks, test and control premolar teeth were extracted under local anaesthesia, placed in Dulbecco's modified Eagle's medium with HAM F12 (1:1 DMEM/HAM F12) and subsequently sectioned vertically through the buccolingual aspect using a high-speed water-cooled diamond bur. The pulps were removed, placed in DMEM/HAM F12 and sectioned horizontally into 1-mm sections. The location of each 1-mm section, from crown to root apex, for each tooth was recorded.

The thoracic aorta used in this assay was dissected from an adult Wistar rat, killed by cervical dislocation. The aorta was placed in DMEM/HAM F12, cleaned, sectioned into 1-mm rings, then each ring halved.

Each 1-mm section of dental pulp, from either an orthodontic treated or control tooth, was embedded with a half ring of rat aorta, placed 1 mm apart, in collagen gel (rat tail type 1) supported by an agarose ring. A number of half rings of rat aorta were embedded alone in collagen gel supported by an agarose ring, forming the control rat aorta group. Each gel was surrounded by serum free DMEM/HAM F12 medium supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) and incubated at 37ºC and 5 per cent CO_2 in a humidified atmosphere. After 24 hours the collagen gels were released from the supporting agarose rings and allowed to float freely in the serum-free media. The medium was changed every 3 days and the gels kept in the humidified incubator for up to 4 weeks. Each co-culture of pulp and rat aorta and culture of rat aorta alone was examined daily for angiogenic changes in the form of microvessel growth.

Growth of microvessels for each explant was measured as described by Derringer *et al.* (1996): (a) quantitatively; by counting the numbers of microvessels using direct observation under brightfield phase inverted microscope (explants

were coded to prevent knowledge of their source at time of counting); and (b) qualitatively; by instantaneous video prints to record and assess growth changes [microscope attached to CCD video camera module connected to a video monitor (Panasonic) and videographic printer (Sony)]. Sequential video prints were used to monitor growth in all the cultures, and in the co-cultures they were used to trace the origin of large vessels formed in the later stages of the study in the area between the pulp and rat aorta.

Identification of microvessels

Light microscopy. Explant cultures were examined for microvessels by brightfield phase inverted microscopy.

Electron microscopy. Identification of microvessels seen by light microscopy was confirmed using electron microscopy. Six sample explant gels were fixed in 4 per cent formaldehyde, 5 per cent glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 48 hours, and post-fixed in 1 per cent osmium tetroxide in 0.1 M phosphate buffer pH 7.4 for 2 hours, then dehydrated through graded ethanols and embedded in araldite resin. Blocks were trimmed and sectioned at 2 μm on an ultramicrotome. Sections were stained with 1 per cent toluidine blue in 1 per cent borax, and mounted on araldite and examined. Ultra-thin sections were cut by ultramicrotome, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

Immunohistochemistry. Immunohistochemistry of sample explants from co-cultures of pulp and rat aorta was used for further confirmation that the outgrowths observed in the explants were microvessels. Staining for factor VIII-related antigen marker for endothelial cells was used. All materials for immunohistochemistry were obtained from Dako, High Wycombe, UK. Initially, polyclonal rabbit anti-human Von Willebrand's factor was used to identify outgrowths from the explants as microvessels. Polyclonal rabbit anti-human Von Willebrand's factor reacts with Von Willebrand's factor in endothelial cells in microvessels from both rat aorta and human pulp, showing a brown granular pattern of reactivity with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Microvessels of pulpal origin were identified by using monoclonal mouse anti-human Von Willebrand's factor which reacts with Von Willebrand's factor (detected by DAB) in endothelial cells in microvessels from human pulp only.

For this part of the study, sample explant gels were fixed in formal calcium (10 per cent formalin, 2 per cent calcium acetate) and processed for routine paraffin sections. Paraffin sections 4 μm thick were cut and placed on silane-coated slides, as for routine histological examination. These slides were deparaffinized and rehydrated, and then incubated for 5 minutes with 3 per cent hydrogen peroxide in distilled water. They were rinsed in water, equilibrated in distilled water in an incubator for 15 minutes and then trypsinized (in 0.1 per cent trypsin, 0.1 per cent calcium chloride pH 7.8) for times ranging from 10 to 30 minutes. They were next rinsed with distilled water, placed in Tris buffered saline (TBS) for 5 minutes, then incubated with normal goat serum 1:5 in TBS for 20 minutes, after which serum was tapped off. The slides were then incubated in sequence with each of the following for 20–30 minutes: polyclonal rabbit anti-human Von Willebrand's factor, dilution 1:200 (code no. A082), biotinylated goat antibody and streptABComplex/HRP duet mouse/rabbit (code no. K492). Between each incubation, slides were rinsed with TBS then placed in the TBS bath for 5 minutes. The slides were incubated for 10 minutes with the chromogenic substrate (DAB) to reveal the immunoreactive sites. They were then rinsed in water, counterstained using Mayer's haematoxylin, dehydrated, cleared in xylene, and mounted in DPX.

The above method was repeated with further sample explants using monoclonal mouse anti-human Von Willebrand's factor, dilution 1:50 (code no. M616) in place of polyclonal rabbit anti-human Von Willebrand's factor. Controls were included omitting the primary antibody in both cases.

Results

The angiogenic changes were compared in the co-cultures of rat aorta and pulp from

orthodontically moved teeth and control teeth, and in the cultures of rat aorta alone. Examination of microvessels was both quantitative and qualitative.

Quantitative examination

Outgrowths of microvessels were observed in all groups of tissue culture within 5 days, reaching a maximum number at around day 10 of culture. The results showed a marked increase in the number of microvessels from both rat aorta and pulp in the orthodontic co-cultures compared with the control co-cultures and the control rat aorta alone culture.

Examining pulp microvessel growth, when compared at days 5, 10, and 14 of culture, the number of microvessels was significantly greater ($P < 0.001$ Wilcoxon signed rank test) in the orthodontic pulp co-cultures than in the control pulp co-cultures (Figure 1, Table 1). Rat aorta microvessel growth, when compared at days 5, 10, and 14 of culture, again showed significantly greater ($P < 0.001$ Wilcoxon signed rank test) numbers of microvessels from the rat aorta in the orthodontic co-cultures compared with the control co-cultures and control cultures of rat aorta alone (Figure 2, Table 1). However, there were

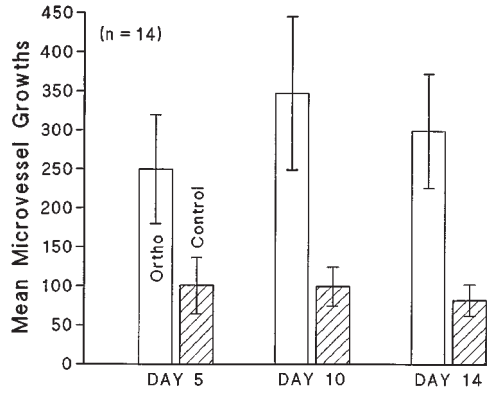

Figure 1 Comparison of the mean number and SEM of pulp microvessel growth in orthodontic and control co-cultures at days 5, 10, and 14 of culture. All differences between orthodontic and control groups were significant. $P < 0.001$ Wilcoxon signed rank test.

Table 1 Comparison of mean number and SEM of (a) total pulp microvessel growths and (b) total rat aorta microvessel growths in orthodontic and control co-cultures, and control rat aorta alone cultures at days 5, 10, and 14 of culture using Wilcoxon signed rank test.

	Day 5		Day 10		Day 14	
	Mean	SEM	Mean	SEM	Mean	SEM
(a) Pulp growth						
Ortho co-culture	250	±70	347	±98	299	±73
Control co-culture	101	±36	99	±25	82	±20
(b) Rat aorta growth						
Ortho co-culture	122	±13	147	±21	146	±26
Control co-culture	50	± 7	51	± 8	48	±11
Rat aorta alone	40	± 6	39	± 7	30	± 7
Wilcoxon signed rank test						
(a) Pulp growth						
Ortho co-culture						
Control co-culture	***		***		***	
(b) Rat aorta growth						
Ortho co-culture						
Control co-culture	***		***		***	
Ortho co-culture						
Rat aorta alone	***		***		***	
Control co-culture						
Rat aorta alone	NS		NS		NS	

***Significant at $P < 0.001$; NS not significant.

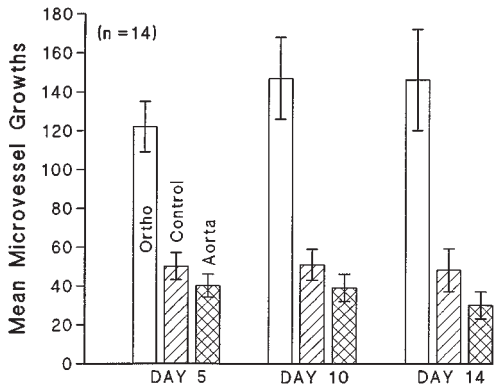

Figure 2 Comparison of the mean number and SEM of rat aorta microvessel growth in orthodontic and control co-cultures, and control rat aorta alone cultures at days 5, 10, and 14 of culture.

no significant differences in microvessel growth between the control co-culture of rat aorta and the control rat aorta alone cultures.

Qualitative examination

Light microscopy. Each explant was monitored daily by light microscope for growth of microvessels from the pulp and rat aorta co-cultures, and the rat aorta alone cultures. Under a bright-field phase-inverted microscope, microvessels were identified on the basis of their morphological features (Nicosia and Ottinetti, 1990). Sequential video prints were used to record growth and morphological changes in the microvessels, and to trace the origin of microvessels in the co-cultures. Microvessel growth was observed in all cultures within a few days and continued in length to a peak at around 10 days. Some microvessels continued to increase in thickness and length, particularly in the orthodontic co-cultures. By 14 days, degeneration of some microvessels began, while other large well-formed microvessels remained, again mainly in the orthodontic co-cultures. By 21 days, most microvessels showed degeneration.

Examining differential growth in the co-cultures, microvessels were observed radiating out from both the rat aorta and pulp sections (Figure 3). Growth on the surfaces facing each other of these sections in the co-cultures appeared more dense than on the other surfaces. This was particularly evident in the orthodontic co-cultures where dense networks of microvessels formed in the area between the rat and pulp explant sections. Under higher magnification, capillary loops with distinct 'tube-like' appearances were observed running between the explant sections (Figure 3).

Electron microscopy. On examination by electron microscopy, the presence of a lumen confirmed the capillary vessel-like appearance. In the co-cultures of rat aorta and orthodontic pulp, small fine vessels were observed in the areas close to the explant sections, while large well-formed vessels were observed in the central area between the explant sections (Figure 4).

Immunohistochemistry. Further confirmation that the outgrowths observed in the explants were microvessels was obtained by immunohistochemistry using staining for factor VIII related antigen marker for endothelial cells. Polyclonal rabbit anti-human Von Willebrand's factor reacted with Von Willebrand's factor in endothelial cells in microvessels radiating out from both rat aorta and dental pulp explants, showing a brown granular pattern of reactivity with DAB. Monoclonal mouse anti-human Von Willebrand's factor reacted with Von Willebrand's factor (again showing a brown granular pattern of reactivity with DAB) in the endothelial cells in microvessels radiating out from human pulp explants only, whereas endothelial cells in microvessels from the rat aorta explants remained unstained (Figure 5). The controls for the polyclonal and monoclonal antibodies did not show a brown granular pattern of reactivity with DAB.

Discussion

A significant increase in microvessel outgrowth of the pulp explants from orthodontically-treated teeth compared with those from control teeth confirms the initial findings (Derringer *et al*., 1996), indicating an increase of pulpal angiogenic growth factors in response to orthodontic force.

The significant increase in microvessel growth from the rat aorta in the orthodontic co-cultures

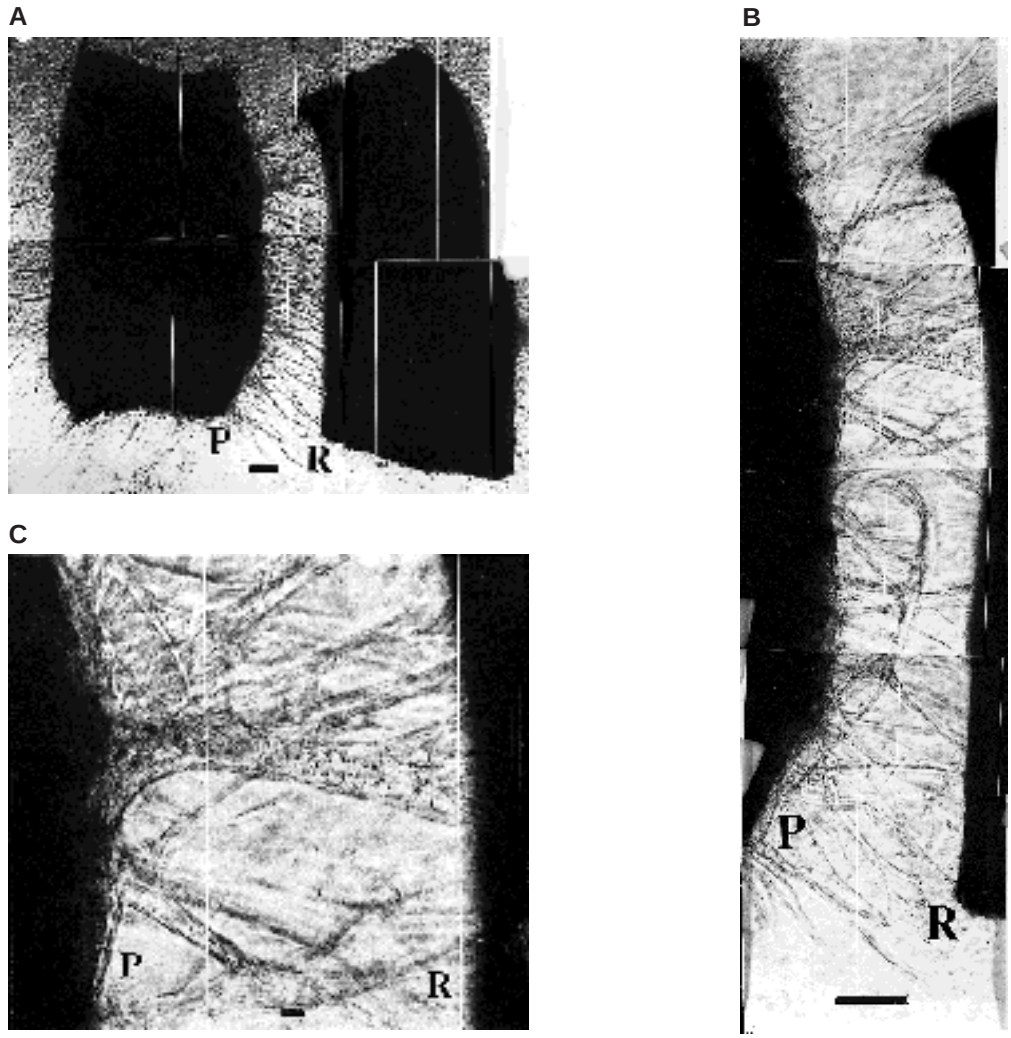

Figure 3 Light microscopic appearance of orthodontic pulp and rat aorta co-culture at day 14 showing microvessels radiating out from the explants, and dense networks of microvessels in the area between the explants from pulp and rat aorta. A collage of video prints at (A) low magnification (bar = 0.1 mm) and (B) higher magnification (bar = 0.1 mm) of area between explants. (C) High magnification of one area (bar = 0.01 mm). P = pulp explant, R = rat aorta explant.

compared with the control co-cultures and control cultures of rat aorta alone, reveals that angiogenic factors released by the pulp are diffusible and capable of stimulating an angiogenic response in the rat aorta. There were no significant differences in microvessel growth between the control co-culture rat aorta and the control rat aorta alone culture, although growth was slightly higher in the co-culture. This indicates that the level of angiogenic growth factors in the control pulp was not significant enough to induce an angiogenic response in the rat aorta. The rat aorta assay has been reported (Nicosia and Ottinetti, 1990) as a method of demonstrating the presence of angiogenic agents. The response of the rat aorta used in this co-culture study therefore demonstrates the presence of diffusible angiogenic growth factors from the dental pulp.

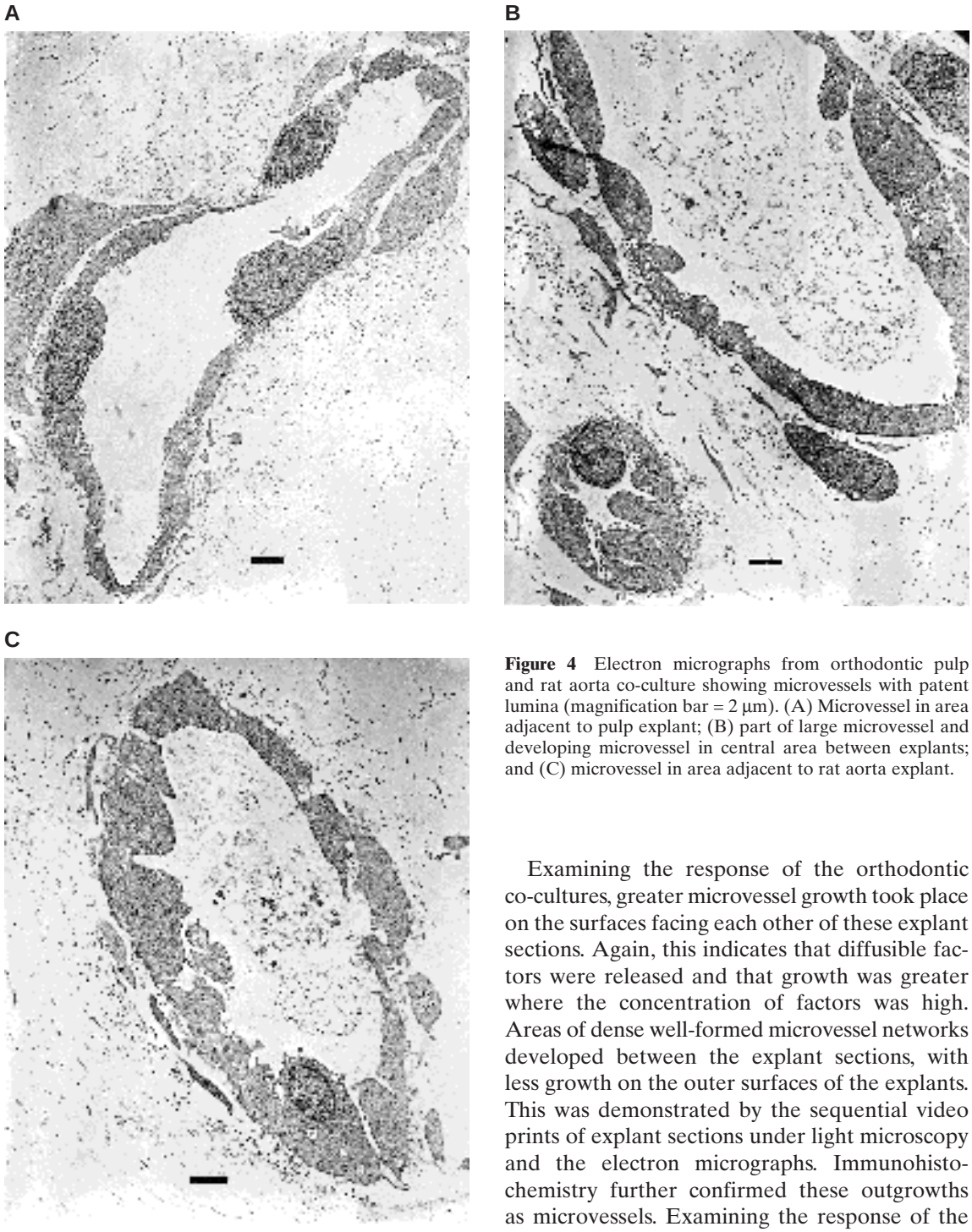

Figure 4 Electron micrographs from orthodontic pulp and rat aorta co-culture showing microvessels with patent lumina (magnification bar = 2 μm). (A) Microvessel in area adjacent to pulp explant; (B) part of large microvessel and developing microvessel in central area between explants; and (C) microvessel in area adjacent to rat aorta explant.

Examining the response of the orthodontic co-cultures, greater microvessel growth took place on the surfaces facing each other of these explant sections. Again, this indicates that diffusible factors were released and that growth was greater where the concentration of factors was high. Areas of dense well-formed microvessel networks developed between the explant sections, with less growth on the outer surfaces of the explants. This was demonstrated by the sequential video prints of explant sections under light microscopy and the electron micrographs. Immunohistochemistry further confirmed these outgrowths as microvessels. Examining the response of the control co-cultures and control aorta culture, growth was more evenly distributed around the explant sections.

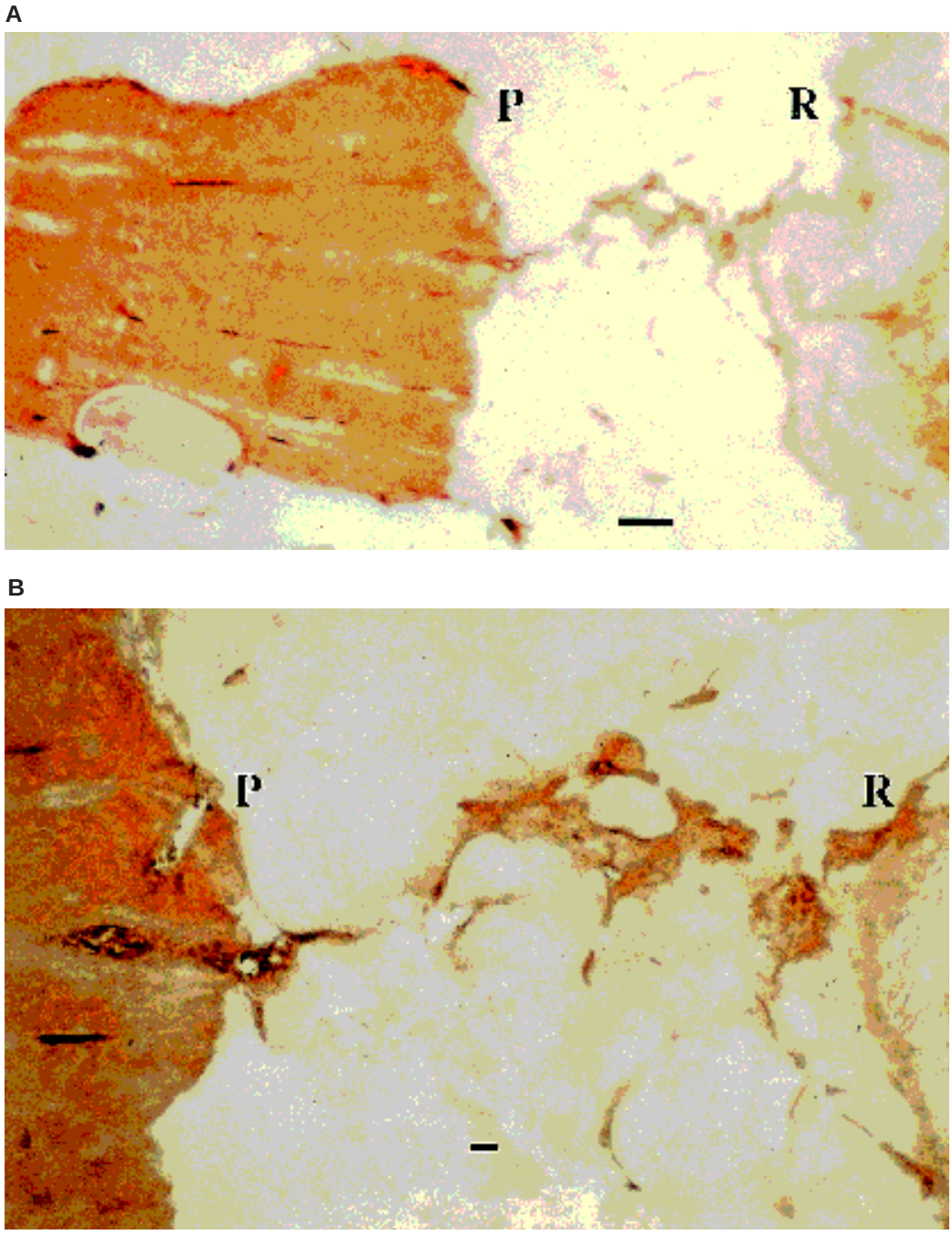

Figure 5 Light micrographs of orthodontic pulp and rat aorta co-culture showing immunohistochemistry appearance using (i) polyclonal rabbit anti-human Von Willebrand's factor which reacted with Von Willebrand's factor in endothelial cells in microvessels from both rat aorta and dental pulp showing a dark brown granular pattern of reactivity with DAB at (A) low magnification (bar = 0.1 mm) and (B) high magnification (bar = 0.01 mm) and using (ii) monoclonal mouse anti-human Von Willebrand's factor which reacted with Von Willebrand's factor in endothelial cells in microvessels in human pulp only showing a dark brown granular pattern of reactivity with DAB at (C) low magnification (bar = 0.1 mm) and (D) high magnification (bar = 0.01 mm). P = pulp, R = rat aorta.

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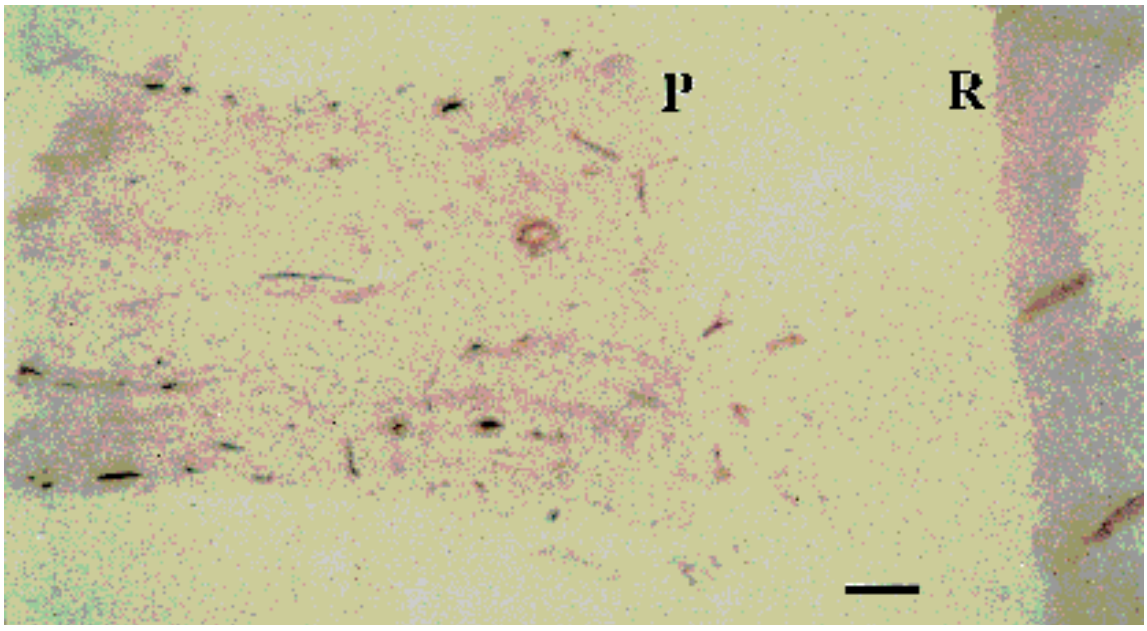

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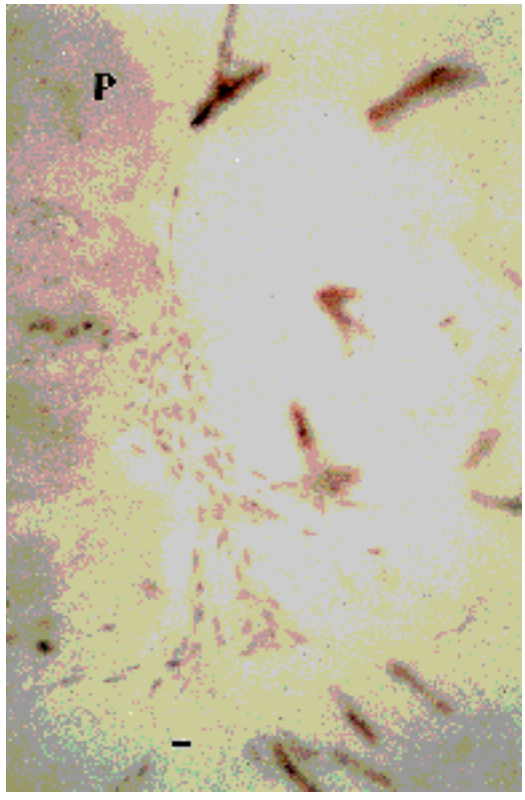

In all cases there was individual variation in both patient and rat aorta response. To avoid the problem of individual patient response, intra-patient untreated teeth were used as controls. Problems of individual rat aorta response were avoided by using the same rat aorta for each patient's orthodontic and control co-cultures, and control rat aorta culture.

This study examined the clinical situation during the early stages of fixed appliance orthodontic treatment by following the effects on the dental pulp of 2 weeks of light, multidirectional forces using initial archwires. The results are therefore of clinical relevance.

The application of orthodontic forces used with fixed appliances involves cellular strain, direct tissue damage, inflammatory changes, and wound healing. These situations are likely to involve the release of angiogenic growth factors in the pulp. Force-induced cellular strain stimulates release of vasoactive neuropeptides causing capillary permeability, leukocyte migration, and the release of inflammatory mediators and growth factors. Following orthodontic tooth movement, substance P (SP) (Nicolay *et al.*, 1990) and vasoactive intestinal polypeptide (VIP; Motakef *et al.*, 1990) have been located in cat pulp, and calcitonin gene-related peptide (CGRP) (Kvinnsland and Kvinnsland, 1990) has been located in rat pulp. Possible links between vasoactive neuropeptides and tumour necrosis factor alpha (TNF-α) have been proposed (Haegerstrand *et al.*, 1990). A number of vaso-active neuropeptides may play a part in neovascularization; in investigation of CGRP, VIP, neurokinin A (NKA) and neuropeptide Y (NPY), only CGRP was found to stimulate proliferation of endothelial cells (Haegerstrand *et al.*, 1990).

Direct tissue damage leads to platelet release from injured vessels and initiation of inflammatory and wound healing processes. A number of polypeptide growth factors have been implicated in the initiation of the angiogenic response and in regulating endothelial cell proliferation in wound healing [basic fibroblast growth factor (bFGF); Terranova *et al.*, 1987, 1989; Tweden *et al.*, 1989; platelet-derived growth factor (PDGF), insulin-derived growth factor (IGF-1), epidermal growth factor (EGF), transforming growth factors (TGF-α and TGF-β), Schultz and Grant, 1991] and in hypoxic and ischaemic conditions in the body [vascular endothelial cell growth factor (VEGF), Harik *et al.*, 1995]. It is possible that some of these factors may therefore participate in an angiogenic response of the pulp to orthodontic force application. Regulation of angiogenesis is by a balance of factors which may be stimulators or inhibitors. Increased angiogenic activity may therefore result from an increase in positive factors or removal of negative regulatory factors.

Conclusions

The results of this study indicate that diffusible angiogenic growth factors released from the pulp, in response to orthodontic force, can induce angiogenesis in other tissues. This study deals with the angiogenic response of pulp from teeth after an initial period of movement with orthodontic fixed appliances. A course of orthodontic treatment involves many months of repeated force activation, and possibly repeated angiogenic stimulation and response of the pulp. Further research will evaluate these changes and elucidate the role of angiogenic growth factors in orthodontic tooth movement.

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Acknowledgements

We wish to thank Dr M. A. Bishop of the Department of Anatomy, Queen Mary and Westfield College, London, UK, for carrying out the electron microscopy part of this study, and K. Paterson and R. Hartley of the Department of Oral Pathology, King's College School of Medicine and Dentistry, London, UK, for carrying out the immunohistochemistry.

This study was financially supported in part by a grant from the research committee of the Biochemical Science Division, King's College, University of London, UK.

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