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Epidermal growth factor released in human dental pulp following orthodontic force

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SUMMARY This study investigated the role of human epidermal growth factor (EGF) in the angiogenic response of the dental pulp to orthodontic force. The release of angiogenic growth factor EGF in human dental pulp following orthodontic force application was examined using neutralizing antibody anti-human (anti-h) EGF to block its effects. The dental pulps from 10 premolar teeth from 10 patients (equal numbers of males and females aged 11–14 years), treated with a straightwire fixed appliance for 2 weeks and extracted for orthodontic reasons, were divided vertically, and sections from each half-pulp were individually co-cultured with a section of rat aorta in collagen surrounded by growth media. Anti-h EGF was added to the media of the co-cultures from one-half of each pulp from each tooth from each patient; the remaining co-cultures from the other half of each pulp without anti-h EGF were used as the controls. Cultures were examined daily by light microscopy for angiogenic growth and number of microvessels.

The addition of anti-h EGF to the growth media in the co-cultures resulted in a significant reduction ($P < 0.05$, Wilcoxon signed rank test) in pulpal and rat aorta microvessel numbers, compared with the control co-cultures. The results indicate that EGF released following orthodontic force application plays a part in the angiogenic response of the pulp.

Introduction

Human epidermal growth factor (EGF) *in vitro* is a mitogen for fibroblasts and endothelial cells, and *in vivo* EGF promotes angiogenesis (Schreiber *et al.*, 1986). EGF has been isolated in low concentrations from human dentine (Roberts-Clark and Smith, 2000). Significantly higher levels of EGF have been found in gingival crevicular fluid following orthodontic force in humans (Uematsu *et al.*, 1996). Orthodontic forces also increased EGF concentrations in paradental cells in human subjects (Sismanidou *et al.*, 1996), and in the cat (Guajardo *et al.*, 2000). EGF, therefore, appears to have a role in orthodontic tooth movement. Its presence and role in human dental pulp in response to orthodontic force requires investigation.

EGF and its receptors (reviewed in Pimental, 1994) have been localized in the dental pulp in the rat (Davideau *et al.*, 1995; Kawase *et al.*, 1995; Wise *et al.*, 1992, 1996), and adjacent to the pulp tissue in humans (Tanikawa and Bawden, 1999). EGF has been found to stimulate DNA synthesis and proliferation of the dental pulp cells in the rat and bovine pulp *in vitro* (Liang *et al.*, 1992; Nakashima, 1992), and in cultures of human pulp cells (Shiba *et al.*, 1998). EGF produced a dose-dependent stimulation in the rate of cell division in rat clonal dental pulp cell line (Kawase *et al.*, 1995).

EGF has been detected in endothelium (Carpenter and Wahl, 1990), where its secretion during endothelial cell proliferation and maturation activates the pericytes through a pathway of cytoplasmic interdigitation between endothelial cells and pericytes (Wakui 1992; Wakui *et al.*, 1993; Chang *et al.*, 1997).

Angiogenesis, the formation of new blood vessels, and continuous adjustment of vessel networks in response to functional needs is necessary throughout life for the development and maintenance of the vascular system (Moore, 2002; Trubiani *et al.*, 2003). Angio-adaptation depends on the interplay of vascular responses to growth factors (Moore, 2002; Trubiani *et al.*, 2003), with endothelial cells playing a key role in the dental pulp response to physical and chemical stimuli (Sawa *et al.*, 1998; Sumpio *et al.*, 2002). Inflammatory stimuli have profound effects on endothelial cells, promoting growth factor synthesis. Interaction between mechanical stress and the EGF/EGF-receptor system in human periodontal ligament cells *in vitro* has been reported (Matsuda *et al.*, 1998). Both inflammatory stimuli and mechanical stress occur in response to orthodontic force application (Davidovitch, 1995).

Previous studies have found angiogenic changes in the human dental pulp in response to orthodontic force (Derringer *et al.*, 1996), and indicate that a combination of diffusible angiogenic growth factors are released which are capable of inducing angiogenesis in other tissues (Derringer and Linden, 1998, 2003). To identify the role of individual growth factors in the pulpal response to orthodontic force, the effect of each angiogenic factor alone and in combination requires investigation. To test which angiogenic growth factors were associated with increased angiogenic response of the pulp to orthodontic force, neutralizing antibodies

against the factors have been used, and reduction of the angiogenic response indicated the involvement of the factor. Of the five angiogenic growth factors previously examined in combination [vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF β) and EGF; Derringer and Linden, 2003], only the effects of four factors (VEGF, FGF2, PDGF, and TGF β) could be considered simultaneously in each patient, as only four teeth per patient were available for use (Derringer and Linden, 2004), leaving the role of EGF to be investigated.

The aim of this study, therefore, was to examine the release of angiogenic growth factor EGF in human dental pulp following orthodontic force application, by using neutralizing growth factor antibody, anti-human (anti-h) EGF to block its effects.

Materials and methods

All materials were purchased from Sigma Chemical Company, Poole, Dorset, UK unless otherwise stated.

Human dental pulp and rat aorta co-culture assay

Following approval by the research ethics committee at King's College Hospital, and informed parental consent, 10 maxillary second premolar teeth from 10 orthodontic patients requiring extractions and fixed appliance treatment were subjected to orthodontic force for a period of 2 weeks. Only teeth free of caries and restorations were included, with equal numbers of male and female patients aged 11 to 14 years. Straightwire brackets (Andrews 0.022 inch; Forestadent, Milton Keynes, UK) were directly bonded (Concise; 3M™, Bracknell, Berks, UK) to upper and lower teeth from second premolar to second premolar teeth, and bands cemented (Ketac; 3M™) to all first molar teeth. Brackets on the test teeth were carefully positioned so that archwires (0.015-inch multistrand stainless steel; Forestadent) gave an active force (in the range of 0.5–1.0 N in a mesial and extrusive direction). After 2 weeks, the test tooth from each patient was extracted under local anaesthesia, and sectioned vertically using a high-speed, water-cooled diamond bur. The pulp was carefully removed using blunt sterile instruments, placed in media (DMEM/HAM F12), and divided vertically into two halves. Each half-pulp was further sectioned horizontally into 1 mm sections using a sterile scalpel blade, and each section was coded for later identification.

Thoracic aortas dissected from adult Wistar rats, killed by cervical dislocation, were cleaned and sectioned into 1 mm rings (one rat aorta used per patient tooth). A three-dimensional proliferative assay technique was used (Nicosia and Ottinetti, 1990; Derringer and Linden, 1998). Each section of pulp was co-cultured with a half-ring section of rat aorta in collagen gel (rat tail type 1) surrounded by 1 ml of medium (DMEM/HAM F12), supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml). Serum-free media was used to eliminate possible responses due to the serum. A number of half-rings of rat aorta alone were similarly cultured.

Neutralizing assays

Anti-h EGF (R & D Systems, Abingdon, Berkshire, UK) at a final concentration of 10 μg/ml of media was used. The concentration of antibody was determined by the manufacturers to give a minimum of 50 per cent neutralization (concentration of antibody required to give one-half maximum inhibition of cytokine activity under the guidelines given by the manufacturer). To each patient's test tooth pulp, anti-h EGF was added to the media of the co-cultures from one-half of each pulp, while co-cultures from the other half were cultured without anti-h EGF, and acted as an intra-tooth control. This would determine if a reduced angiogenic response occurred with the addition of anti-h EGF. Prior to this investigation, no significant reduction in microvessel numbers had been found when anti-h EGF was added individually or in combination with other neutralizing antibody growth factors to rat aorta cultures alone (Derringer and Linden, 2003). Therefore, in this study the remaining sections of rat aorta were cultured alone, allowing comparison with the co-culture rat aorta both with and without the addition of anti-h EGF. All cultures were incubated at 37°C and 5 per cent carbon dioxide in a humidified incubator for 2 weeks. The media (either with or without anti-h EGF) was changed every 4 days.

Examination of microvessels

Each co-culture of pulp and rat aorta and culture of rat aorta alone was examined daily for angiogenic response in the form of microvessel proliferation. Microvessel growth was examined quantitatively (numbers of microvessels from each explant counted at days 5 and 10 using a bright-field, phase-inverted microscope, and explants were coded and counts repeated and averaged), and qualitatively evaluated, using sequential video prints for vessel identification and monitoring growth (microscope attached to a CCD video camera, monitor, and printer; Panasonic, Sony; Derringer and Linden, 1998).

Statistical analysis

Data are presented as means and standard errors of the means. Wilcoxon's signed rank test was used to compare the results of the test and control groups.

Results

Quantitative examination

The addition of anti-h EGF to the growth media in the co-cultures resulted in a significant reduction in pulpal microvessel numbers compared with the control half-pulp co-cultures at both day 5 (30 ± 6 versus 39 ± 7) and day 10

(33 ± 8 versus 45 ± 7) of culture (Figure 1A). Differences between the anti-h EGF group and the control group showed a reduced angiogenic response with the addition of anti-h EGF. The mean percentage reduction of microvessel numbers with the addition of anti-h EGF was 23 at day 5 and 26.6 at day 10 of culture. Individual patient variations both in pulpal angiogenic response without anti-h EGF and in the reduction of the angiogenic response with anti-h EGF occurred (Figure 2A,B), with pulp explants from patients 1 and 2 showing a large angiogenic response compared with those from patients 9 and 10, and pulp explants from patients 2, 7, 8, and 9 demonstrating a marked reduction in angiogenic response with anti-h EGF compared with those from patients 3 and 4.

Examining the rat aorta response, the addition of anti-h EGF to the growth media in the co-cultures resulted in a significant reduction in rat aorta microvessel numbers compared with the control co-cultures ($P < 0.016$) at day 5 (18 ± 2 versus 26 ± 2) and ($P < 0.05$) day 10 (17 ± 2 versus 26 ± 3) of culture (Figure 1B). Differences between the experimental and control groups were a reduction in angiogenic response with the addition of anti-h EGF. The mean percentage reduction of microvessel numbers with the addition of anti-h EGF was 30.7 at day 5 and 34.6 at day 10 of culture. Again, variability in response in the co-cultures from individual patients to anti-h EGF was seen (Figure 3A,B).

Microvessel numbers in both rat aorta co-cultures with and without anti-h EGF were significantly greater ($P < 0.05$) at days 5 and 10 of culture, than in the rat aorta-alone cultures (Figures 1b, 3A,B).

Qualitative examination

Within a few days of culture, microvessels were observed in all cultures. Microvessel growth was noticeably more dense, vigorous, and extensive in the pulp and rat aorta explants in the co-cultures without anti-h EGF than in those with anti-h EGF. Growth of microvessels was less dense, vigorous, and extensive in the rat aorta-alone cultures.

Discussion

The results show a clear difference between the groups with and without anti-h EGF added to the media. In the co-cultures, a reduced angiogenic response occurred in the pulps with the addition of anti-h EGF, and a reduced angiogenic response of the rat aorta appeared to occur by neutralization of some diffusible EGF from the pulp. Microvessel numbers in both rat aorta co-cultures with and without anti-h EGF were significantly

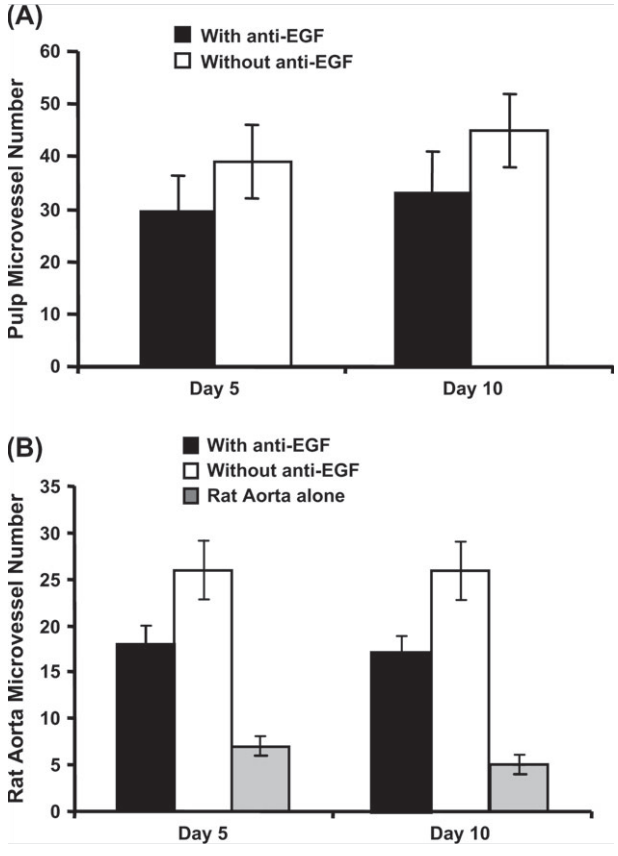

Figure 1 Comparison of the mean and standard error of the mean of (A) pulp microvessel numbers in the co-cultures with and without anti-human epidermal growth factor (anti-h EGF) at days 5 and 10 of culture and (B) rat aorta microvessel numbers in the co-cultures with and without anti-h EGF, and in the rat aorta-alone cultures at days 5 and 10 of culture ($n = 10$).

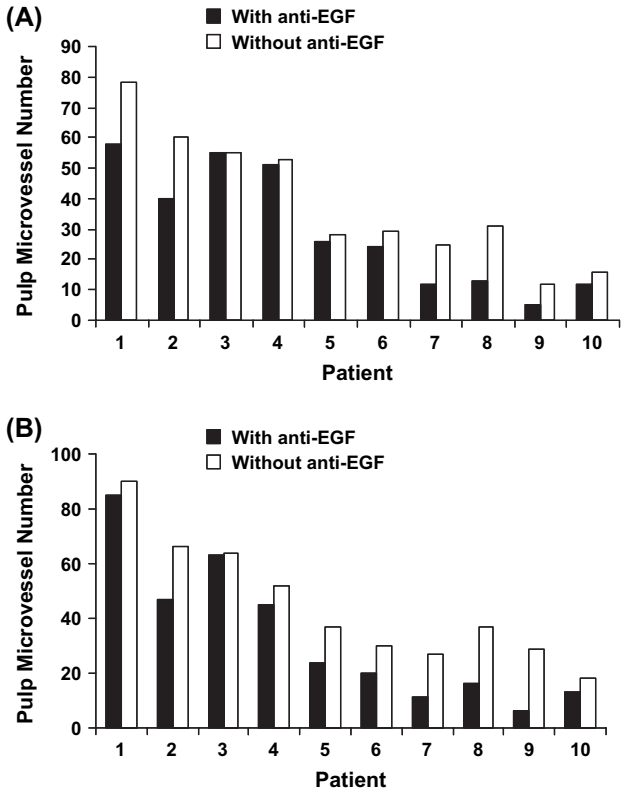

Figure 2 Comparison of pulp microvessel numbers in the 10 individual patient's co-cultures with and without anti-human epidermal growth factor (anti-h EGF) at day 5 (A) and day 10 (B) of culture.

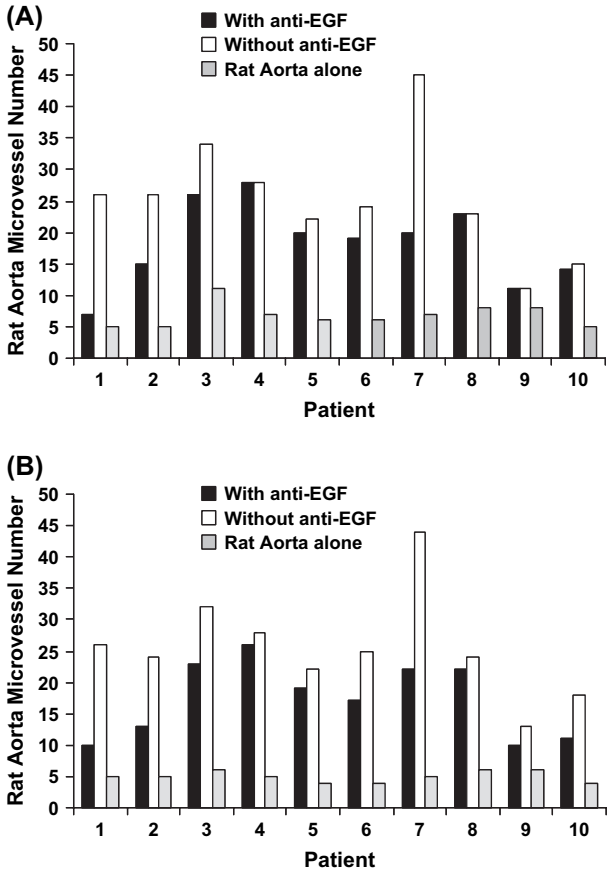

Figure 3 Comparison of rat aorta microvessel numbers in the 10 individual patient's co-cultures with and without anti-human epidermal growth factor (anti-h EGF), and in the rat aorta-alone cultures at day 5 (A) and day 10 (B) of culture.

greater than in the rat aorta-alone cultures. Therefore, although some reduction of angiogenic response was caused by the addition of anti-h EGF, it was not sufficient to reduce the number of microvessels to that in rat aorta alone.

The mean percentage reduction with the addition of anti-h EGF continued to progress from day 5 to day 10 in the co-cultures, showing a continued reduction in angiogenic response caused by the addition of anti-h EGF. Qualitative differences in microvessel growth between the groups on days 5 and 10 of culture also support this view. Qualitative differences in microvessel growth importantly must be viewed in conjunction with the quantitative findings.

The results of this study indicate that EGF is involved in the angiogenic response of the pulp, and support the *in vivo* findings that EGF promotes angiogenesis (Schreiber *et al.*, 1986). EGF has also shown therapeutic potential as an accelerator of angiogenesis (Chang *et al.*, 1997). Orthodontic force has resulted in an increase in EGF levels in human gingival crevicular fluid after 24 hours of force (Uematsu *et al.*, 1996), while upregulation of EGF in the periodontal ligament has been reported in areas of healing following 37 days of force (Sismanidou *et al.*, 1996). The involvement of EGF in the response of human dental pulp to orthodontic force was also confirmed by the results of the present investigation, where 14 days of force was used, corresponding to the clinical situation in the first weeks of fixed appliance orthodontic treatment.

EGF concentration in tissues is generally low (Carpenter and Wahl, 1990), with low concentrations found in human dentine (Roberts-Clark and Smith, 2000). EGF-binding cells have been localized in apical parts of erupting human premolars (Thesleff *et al.*, 1987) and in various oral tissues (Whitcomb *et al.*, 1993). Angiogenic growth factors can have an effect, in combination at lower concentrations than alone, and EGF has been reported to have synergistic reactions with TGF β (Thesleff *et al.*, 1983). Proliferation and differentiation of pulp cells are regulated not only by individual growth factors including EGF but also by factors in combination (Liang *et al.*, 1992).

Individual variations were apparent both in angiogenic response and in the reduction of angiogenic response with the addition of anti-h EGF. Similar inter-patient variation was found in previous studies with VEGF, FGF2, PDGF, and TGF β (Derringer and Linden, 2003, 2004), indicating that variations in individual levels of and response to angiogenic growth factors may give rise to variations in response to orthodontic force from individual to individual.

Age is an important factor to consider. Decreased response to EGF with age results principally from decreased sensitivity to EGF due to a decrease in the number and affinity of high-affinity EGF receptors (Kawamoto *et al.*, 1989). In this study, as in previous research (Derringer and Linden, 2003, 2004), although the variables of age and anatomy were reduced to a certain extent by limiting the age range and teeth used, other variables are still present so that inter-patient differences are still evident. Genetic background (Maltha and Van Leeuwen, 1999), age and genetics (Terranova *et al.*, 1994), and individual morphological and biological differences (Davidovitch, 1995; Davidovitch *et al.*, 1996) can lead to altered individual responses. The variation in an individual patient's angiogenic response, and to the blocking effect caused by anti-h EGF seen in this study, together with the results of previous research (Derringer and Linden, 2003, 2004) support this view.

Conclusions

The results of this study indicate that EGF released following orthodontic force application plays a part in the angiogenic response of the pulp.

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