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Inter- and intra-site heterogeneity in the expression of fetal-like phenotypic characteristics by gingival fibroblasts: potential significance for wound healing

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SUMMARY

We have previously reported that fetal and adult skin fibroblasts display distinctive migratory phenotypes on 3-D collagen substrata and that these behavioural characteristics may be quantified by a function defined as the *cell density migration index* (CDMI). Subsequent work indicated that this difference in migratory phenotype was due to the production by fetal fibroblasts of a *migration stimulating factor* (MSF) that is not produced by normal adult skin fibroblasts. We now present data indicating that: (a) unselected fibroblasts obtained from 14/14 (100%) of adult gingival explants expressed fetal-like CDMI values compared to only 1/10 (10%) of similarly explanted paired skin cells; (b) 12/12 (100%) of these gingival fibroblast lines also produced detectable quantities of MSF compared to 0/9 (0%) of the tested skin cells; (c) by microdissection studies, gingival fibroblasts obtained from different anatomical microdomains consisted of behaviourally distinct subpopulations, with cells derived from the papillary tips (PAP fibroblasts) displaying fetal-like CDMI values and persistent MSF production, whilst cells obtained from the deeper reticular tissue (RET fibroblasts) were adult-like with respect to these two criteria; (d) PAP fibroblasts were also smaller and achieved higher saturation cell densities compared to paired RET cells; (e) PAP fibroblasts passaged in vitro underwent a fetal-to-adult phenotypic transition characterized by the adoption of various RET cell characteristics, including the acquisition of CDMI values falling within the adult range and cessation in MSF production; and (f) early passage PAP fibroblasts incubated in the presence of an affinity-purified anti-MSF rabbit polyclonal antibody were induced to alter their migratory phenotype and exhibited CDMI values falling within the adult range. Statistical analysis indicated a highly significant correlation between the expression of a fetal-like CDMI and production of MSF ($P<0.00001$, using the Fisher exact contingency test). Taken together, these observations suggest that the production of MSF by PAP fibroblasts is responsible for their characteristically fetal-like migratory behaviour. The existence of such inter- and intra-site phenotypic heterogeneity in populations of skin and gingival fibroblasts is discussed in the context of fibroblast lineage relationships and the possible contribution of persistently fetal-like fibroblast subpopulations to connective tissue function in wound healing.

Key words: fibroblast, migration, heterogeneity, migration stimulation factor, gingiva, wound healing

INTRODUCTION

In spite of their similar appearance, fibroblasts are in fact a highly diverse population of cells exhibiting a considerable degree of both inter- and intra-site heterogeneity (as reviewed by Schor and Schor, 1987). Many of the studies documenting such heterogeneity have employed skin and gingival fibroblasts as a consequence of their relative accessibility. Such studies have clearly demonstrated that selected fibroblast subpopulations obtained from both skin (Martin et al., 1974; Harper and Grove, 1979; Tajima and Pinnell, 1981; Azzarone and Marcieira-Coehlo, 1982; Korn, 1985; Korn et al., 1985; Schafer et al., 1985,1989; Goldring et al., 1990) and gingiva (Ko et al., 1977; Hurum et al., 1982; Hassell and Stanek, 1983; Bordin et al., 1984) display significant heterogeneity with respect to such fundamental aspects of cell behaviour as proliferative potential, response to growth factors and matrix biosynthesis. Comparable observations relating to intra-site

fibroblast heterogeneity have been made with cell subpopulations derived from other tissues as well, including periodontal ligament (Limeback et al., 1983), lung (Kondo et al., 1985) and synovium (Brinckerhoff and Nagel, 1981). Clearly defined site-specific differences between skin and gingival fibroblasts have also been reported (Bronson et al., 1988); the existence of such inter-site heterogeneity has been similarly well documented with respect to other anatomical sites (such as foreskin and forearm) and, indeed, failure to recognise these intrinsic phenotypic differences have led to the erroneous interpretation of data in the past (Thompson et al., 1983).

Fibroblasts obtained from the same tissue have also been reported to display developmentally dependent changes in their proliferative potential (Smith et al., 1978; Mollenhauer and Bayreuther, 1986), ability to form colonies in semi-solid medium (Nakano and Ts'O, 1981), cellular response to cytokines (Hill et al., 1986), secretion of matrix-degrading enzymes (Sottile et al., 1989; West et al., 1989; Basset et al., 1990), migratory behaviour (Muggleton-Harris et al., 1982; Albini et al., 1988; Pienta and Coffey, 1990), cytokine production (Stoker and Gherardi, 1991) and matrix deposition (Bartold et al., 1986; Chen et al., 1989; Martin et al., 1990). Transitions in these various characteristics from a fetal to an adult phenotype have been observed to take place both in vivo and as a function of cellular 'ageing' in vitro.

Phenotypic heterogeneity has similarly been documented with respect to fibroblasts derived from healthy and pathological sites of the same tissue, these including healthy and wounded oral mucosa (Bronson et al., 1989) and skin (Vande Berg et al., 1989; Bertolami and Bronson, 1990), normal and keloid dermis (Russell et al., 1988; Harper, 1989; Kischer et al., 1989), and healthy and inflamed gingiva (Larjava et al., 1989; Häkkinen and Larjava, 1992). Skin fibroblasts obtained from patients with various types of cancer have also been reported to differ from their healthy adult counterparts with respect to a number of phenotypic characteristics, including growth potential, synthesis of matrix macromolecules and matrix-degrading enzymes, and cytoskeletal organization (reviewed by Schor et al., 1991).

Our own work in this area has revealed differences between fetal and adult skin fibroblasts with respect to their migratory behaviour on 3-D matrices of collagen fibres (Schor et al., 1985a). Such differences are a consistent feature of cell phenotype in vitro and may be expressed in quantitative terms using the *cell density migration index* (CDMI). Subsequent work indicated that this difference in migratory phenotype was due to the production by fetal fibroblasts of a *migration stimulating factor* (MSF) that is not produced by normal adult skin fibroblasts (Schor et al., 1988a; Grey et al., 1989). MSF has since been purified to apparent molecular homogeneity and characterized in terms of a number of biochemical criteria, including partial amino acid sequence (Schor et al., 1993); these studies indicate that MSF is an apparently novel protein containing an internal domain exhibiting striking amino acid sequence homology with the gelatin-binding domain of fibronectin. The migration-stimulating activity of MSF is neutralized by monoclonal antibodies directed against the gelatin-binding domain of fibronectin, as well as an affinity-purified anti-MSF polyclonal antibody that does not recognise either native fibronectin or its gelatin-binding domain in both ELISA and western blots.

Much of our previous work has been concerned with documenting the presence of fetal-like (MSF-producing) fibroblasts in breast cancer patients. These studies have demonstrated that: (a) tumour-derived fibroblasts obtained from approximately 50% of sporadic breast cancer patients expressed CDMI values falling within the fetal range (Durning et al., 1984; Schor et al., 1985b); (b) paired skin fibroblasts obtained from the same individuals also expressed a fetal-like migratory phenotype, thereby indicating the systemic nature of this stromal cell abnormality (Durning et al., 1984); and (c) skin fibroblasts obtained from approximately 90% of patients with familial breast cancer behaved in a similar fetal-like fashion, as did greater than 50% of their unaffected first-degree relatives (Schor et al., 1986; Haggie et al., 1987). Subsequent work indicated that the fetal-like fibroblasts obtained from breast cancer patients also produced MSF and that this MSF was indistinguishable from that synthesized by fetal fibroblasts (Schor et al., 1988b). In all of these studies, there was an approximate 10% incidence of fetal-like migratory behaviour and corresponding production of MSF by skin fibroblasts obtained from age- and sex-matched healthy controls.

These findings regarding the existence of inter-site, intra-site, developmentally dependent and pathology-related heterogeneity amongst fibroblasts raise a number of interesting questions regarding the origin and possible significance of fibroblast diversity with respect to tissue function. With this in mind, the objective of this study has been to compare fibroblasts obtained from different microregions of gingiva with skin fibroblasts obtained from the same donor in terms of migratory phenotype and MSF production. Gingival fibroblasts were chosen because of their previously demonstrated phenotypic diversity and ease of dissection into papillary (PAP) and reticular (RET) microregions. Data are presented in this paper indicating that: (a) gingival fibroblasts obtained by nonselective explant culture from 100% (14/14) of the donors examined displayed a fetal-like CDMI and produced MSF compared to only 10% (1/10) of matched skin cells obtained by the same explant technique; and (b) only PAP fibroblast subpopulations expressed these fetal-like characteristics, whereas the larger and more slowly growing RET cells derived from the deeper reticular tissue behaved in a typically adult-like fashion. These data documenting the existence of both inter-and intra-site heterogeneity amongst gingival fibroblasts are discussed in the context of fibroblast lineage relationships and the possible contribution of distinct fibroblast subpopulations to tissue function.

MATERIALS AND METHODS

Cells and culture conditions

Healthy gingival tissue was obtained from 14 patients undergoing routine dental surgery in either the Department of Periodontics or the Department of Oral Surgery, University of Manchester Dental Hospital. Samples of oral mucosa derived from the cheek and the floor of the mouth were also obtained from three further patients and included in this study. A 2 mm pinch biopsy of forearm skin was obtained from certain of these patients after informed consent. Details relating to the age, sex, type of oral mucosal tissue obtained, and availability of matched skin cells, are provided in Table 1.

Gingival tissue was washed once in Hanks' balanced salt solution (Gibco, Paisley, Scotland) and immediately chopped into 2 mm cubes.

Table 1. Data relating to the donors and site of oral mucosal biopsy

Donor	Age	Sex	Oral site
156	66	F	Gingiva
158	64	M	Gingiva
204	44	M	Gingiva
224	49	M	Gingiva
226	22	F	Gingiva
238	19	M	Gingiva
264	16	F	Gingiva
271	49	F	Gingiva
310	19	M	Gingiva
314	41	F	Gingiva
315	28	F	Cheek
317*	32	M	Gingiva
324	42	F	Cheek
334*	38	M	Gingiva
339*	30	F	Gingiva
392	66	M	Floor of mouth
438*	48	F	Gingiva

Matched forearm skin and oral mucosal biopsies were obtained from the indicated donors.

*Donors from whom only an oral mucosal biopsy was taken.

Several methods were used to isolate distinct fibroblast populations from this tissue. Unselected normal oral mucosal (NOM) fibroblasts were obtained from gingival, cheek and floor of mouth tissue specimens using standard explant techniques (Schor et al., 1985a). Selected subpopulations of fibroblasts derived from the papillary (PAP) and reticular (RET) regions of the gingiva were additionally obtained from three patients using the following protocol. The 2 mm cubes of gingiva were placed in 5 ml of 0.25% trypsin in phosphate buffered saline for 16 hours at 4°C, followed by a 30 minute incubation at 37°C. Using a dissecting microscope, the epithelium was gently teased away from the underlying lamina propria, thus revealing the projecting connective tissue papillae. Groups of from 1-3 papillae, as well as similarly sized pieces of deeper reticular tissue, were dissected free, embedded in 0.5 ml collagen gels cast in 24-well dishes (Gibco) and overlaid with MEM growth medium (Gibco) supplemented with 15% aseptic fetal calf serum (FCS), glutamine, non-essential amino acids, sodium pyruvate and streptomycin (Schor, 1980). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air until a significant cellular outgrowth occurred (this varying considerably between PAP and RET fibroblasts); the cells were then harvested by dissolution of the gel with 1 unit/ml bacterial collagenase (cat. no. CLS1; Cambridge Bioscience, Cambridge) and trypsin (Schor, 1980), and the released cells were plated into 90 mm stock tissue culture dishes. Serial subcultivation of all cell types (NOM, PAP and RET) in stock culture was then continued at a split ratio of 1:3 when confluence was achieved; apart from the in vitro ageing studies, only cells between passages 2 and 6 were used in this study.

Skin fibroblasts were grown from the pinch biopsies by the same explant culture technique as used to obtained the unselected gingival fibroblasts. Collagen used in this study was extracted from rat tendons and used to make 3-D gels as previously described (Schor, 1980).

Measurement of cell proliferation and migration

Cell proliferation on 35 mm plastic tissue culture dishes (i.e. standard monolayer cultures) and within 3-D collagen gels cast in 35 mm tissue culture dishes was measured as previously described (Schor, 1980). Cultures were set up at 2×10^4 cells per culture (both on the plastic dish and within the collagen gel) and maintained in MEM containing either high (15%) or low (2.5%) FCS. Cells were harvested and counted using a Coulter electric particle counter at various times over a three-week incubation period. All cell number measurements were made in triplicate.

Fibroblast migration into 3-D collagen gel cultures and calculation of CDMI values were performed as previously described (Schor et al., 1985a). Accordingly, 2 ml collagen gels were cast in 35 mm plastic tissue culture dishes and overlaid with 1 ml of serum-free MEM. Confluent stock cultures of fibroblasts were trypsinized, pelleted by centrifugation and resuspended in growth medium containing 20% fetal calf serum. This cell suspension was then used to prepare a high and a low cell density plating inoculum; the cell count in the high density inoculum was adjusted so that plating 1 ml gave 2.5×10^5 cells per gel (i.e. confluent density), whereas that in the low density inoculum was adjusted to give 10^4 cells per gel (i.e. subconfluent density). Considering the 2 ml volume of the collagen gel, the 1 ml serum-free medium overlay and the 1 ml cell inoculum, this procedure gave a final concentration of 5% serum. Cultures were incubated for four days and the percentage of fibroblasts found within the 3-D gel matrix was then determined by counting the number of cells on the collagen gel surface and within the collagen matrix in 10-15 randomly selected fields using a Leitz Labovert microscope (Schor 1980). Replicate gels were counted for each point. These results were then used to calculate the CDMI for each cell line according to the following definition:

$$\text{CDMI} = \log [\%\text{LD mig}/\%\text{HD mig}] ,$$

where %LD mig is the percentage of cells within the gel matrix in low density cultures and %HD mig is the corresponding value for cells plated at high density. In accordance with our previous studies (Schor et al., 1985a,b, 1986; Haggie et al., 1987), fibroblasts with CDMI values greater than +0.4 were classified as displaying an adult-like migratory phenotype, whilst cells with CDMI values less than +0.4 were classified as fetal-like.

Determination of MSF production by cultured fibroblasts

Confluent cultures of skin and gingival fibroblasts in 90 mm plastic tissue culture dishes were washed 5× with serum-free culture medium (SF-MEM) and then incubated with 5 ml SF-MEM for 72 hours in a humidified, gassed incubator at 37°C. The resulting conditioned medium (CM) was collected and passed through a 0.22 μm Millipore filter to remove any cellular debris. The presence of migration-stimulating activity in these samples was determined by overlying collagen gel substrata with 1 ml of neat CM (instead of SF-MEM) in the cell migration assay described above. CM samples were considered to contain MSF if the stimulation of migration achieved was greater than 1.8-fold that of control assay cultures incubated with SF-MEM (Picardo et al., 1991).

In other experiments, CM samples were first fractionated by Pharmacia FPLC gel filtration chromatography (using a Superose 12 column), as previously described (Schor et al., 1988a). Fractions of 1 ml were collected and dialysed against SF-MEM for 48 hours at 4°C, and subsequently tested for the presence of MSF activity as described above for unfractionated CM.

Morphometry, histology, electron microscopy and immunolocalization of MSF

Fibroblast size was estimated by projecting photographic negatives at a magnification of ×3.5 and measuring the long axis of the cell with a ruler.

Gingival tissue to be utilized for histology was fixed for 24 hours in 10% neutral buffered formalin and then processed into paraffin wax using routine histological preparatory procedures. Sections were cut at a thickness of 5 μm and stained using haematoxylin and eosin.

Tissue samples to be processed for scanning electron microscopy were fixed for 6 hours in half-strength Karnovsky's fixative, washed in 0.1 M cacodylate buffer and then briefly rinsed in distilled water. Following progressive dehydration in a graded ethanol series, specimens were critical point dried from carbon dioxide in wire

baskets. Dried specimens were fixed to Cambridge stubs and coated with 40-50 nm of gold using a Polaron sputter coater, after which they were examined in a Cambridge 400 scanning electron microscope.

Fresh gingival tissue to be used for immunolocalization of MSF was snap frozen in isopentane cooled by liquid nitrogen. The tissue was mounted in OCT and frozen sections were prepared at 4 μm using a Leitz rotary cryostat. Sections were washed for 30 minutes in phosphate buffered saline and incubated for 30 minutes in an affinity-purified anti-MSF rabbit polyclonal antibody at a dilution of 1:10 (1 μg/ml) in a humidified chamber (Schor et al., 1993). Following two further washes in phosphate buffered saline, sections were labelled with FITC-conjugated goat anti-rabbit secondary antibody (Dako, High Wycombe) at 1:50 for 30 minutes and mounted in Dabco viewing solution (Sigma Chemical Co., Poole, Dorset). Control sections were prepared using non-immune serum. Sections were viewed in a Zeiss Axioplan microscope under ultraviolet illumination.

Statistical analysis

Statistical analyses were performed using the 'Instat' computer program (GraphPad Software, San Diego, California).

RESULTS

Migratory phenotype of matched skin and gingival fibroblasts

Normal oral mucosal (NOM) fibroblasts were obtained by unselective explant culture of small pieces of gingiva obtained from 14 healthy donors; matched skin tissue was also obtained from 10 of the donors and used to establish unselected fibroblast lines (SK) using the same explant culture technique. Data are presented in Table 2 regarding the CDMI values expressed by these fibroblast lines, which have accordingly been classified as being either adult-like (A) or fetal-like (F), as previously described (Schor et al., 1985a). These data indicate that 100% (14/14) of the gingival fibroblasts displayed a fetal-like migratory phenotype compared to only 10% (1/10) of the matched skin cells; $P<0.0001$, as calculated by two-tailed *t*-test. These results are in keeping with our previous studies in which skin fibroblasts obtained from approximately 10% of healthy adult donors were found to express CDMI values falling within the fetal range (Schor et al., 1986; Haggie et al., 1987). They further indicate that, in such healthy adults, fibroblasts obtained from another anatomical site (i.e. gingiva) may continue to express a fetal-like migratory phenotype.

Conditioned medium (CM) was prepared from these cell lines and assayed for the presence of MSF (Picardo et al., 1991, 1992). This protocol involves testing unfractionated (neat) CM samples for their ability to stimulate the migration of a standard target adult fibroblast line (see Materials and Methods); relative stimulation greater than 1.8 times the control are considered significant and are indicated in bold in Table 2. The results reveal that all of the gingival fibroblast lines examined (12/12) produced detectable MSF activity, whereas none of the matched skin cells did so (0/9); $P<0.0001$, as calculated by two-tailed *t*-test. Data relating to the CDMI and production of MSF by paired (i.e. obtained from the same donor) skin and NOM fibroblasts are summarized in Fig. 1; statistical analysis indicates a strong correlation between expression of a fetal-like CDMI and MSF production ($P<0.00001$, Fisher exact contingency test).

CDMI values were also obtained with cheek fibroblasts derived from two donors; interestingly, these cells expressed an adult-like migratory phenotype and therefore differed in this respect from their gingival counterparts. Unfortunately, the total number of cheek-derived fibroblasts lines was too small

Table 2. CDMI values and MSF production by matched skin and oral mucosal fibroblasts

	Skin					NOM				
Donor	%LD	%HD	CDMI	Class	Stimul	%LD	%HD	CDMI	Class	Stimul
						Gingiva				
156	11.7	1.9	0.80	A	1.3	8.2	6.2	0.12	F	**2.1**
158	6.5	1.7	0.57	A	1.2	30.1	20.0	0.18	F	**2.7**
204	18.1	4.2	0.63	A	0.9	7.2	5.7	0.10	F	**1.9**
224	13.2	3.3	0.59	A	1.3	13.2	9.3	0.15	F	**2.0**
226	33.5	11.9	0.45	A	1.1	8.9	4.6	0.29	F	**3.1**
238	14.0	3.4	0.62	A	1.1	19.7	10.0	0.29	F	
264	9.7	6.1	0.20	F	1.2	19.9	18.0	0.04	F	**2.2**
271	20.1	4.2	0.68	A		3.8	9.2	–0.38	F	
310	12.1	3.1	0.60	A	1.5	5.6	4.4	0.10	F	**1.9**
314	17.2	4.3	0.60	A	1.2	4.2	4.1	0.01	F	**1.9**
317						7.8	4.6	0.23	F	**2.2**
334						7.9	4.6	0.23	F	**2.5**
339						13.5	10.9	0.09	F	**1.8**
438						4.3	5.0	–0.07	F	**2.6**
						Cheek				
315	24.7	6.1	0.61	A		9.9	3.1	0.51	A	
324	8.6	2.3	0.57	A		13.0	4.4	0.48	A	
						Floor of mouth				
392	20.2	2.6	0.88	A		16.1	11.1	0.16	F	

Data are presented concerning the migration of nonselected explant cultures of normal oral mucosal (NOM) fibroblasts (derived from the gingiva, cheek and floor of mouth) and matched skin cells plated at low density (%LD) and high density (%HD). These cells have been classified (Class) as expressing either an adult-like (A) or fetal-like (F) migratory phenotype on the basis of the calculated CDMI values, as previously described (Schor et al., 1985a). The production of MSF by these cells was assessed as described in Materials and Methods; these data are expressed as the relative stimulation of migration (Stimul) of a target adult fibroblast line, with values greater than 1.8 (shown in bold) being considered as indicative of significant migration stimulating activity (Picardo et al., 1991). The presented data are the respective means of from 2-5 independent experiments.

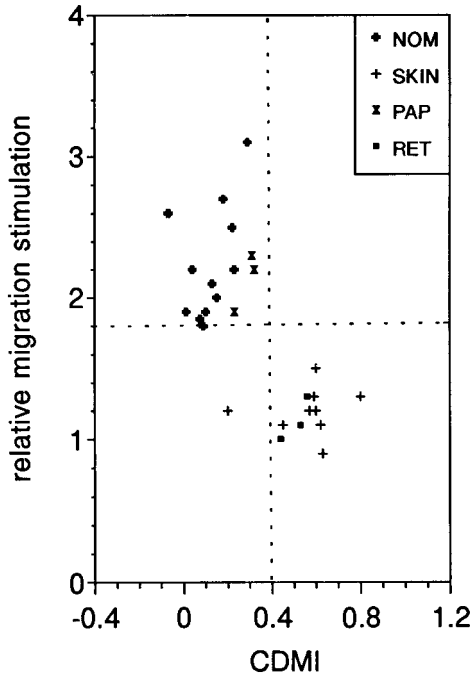

Fig. 1. Correlation between CDMI and MSF production. Data are presented concerning the CDMI and relative stimulation of target cell migration by neat conditioned medium (measure of MSF production) for various skin, gingival (NOM), gingival papillary (PAP) and gingival reticular (RET) fibroblasts. CDMI values greater than +0.4 are regarded as adult-like, whilst values smaller than this are regarded as fetal-like; a relative stimulation of target cell migration greater than 1.8 is regarded as indicative of significant MSF in the conditioned medium (Picardo et al., 1991).

to draw any meaningful conclusions regarding the general validity of this observation. One unselected fibroblast line was also established from tissue derived from the floor of the mouth; this consistently gave CDMI values falling within the fetal range.

Characterization of isolated PAP and RET gingival fibroblasts

The following experiments were done in order to characterize fibroblasts derived from different microregions of gingiva in terms of a number of criteria, including morphology, growth dynamics, migratory phenotype and production of MSF. The objective of this work was to ascertain whether there was any: (a) intra-site heterogeneity with respect to the tissue distribution of MSF-producing fibroblasts in gingiva; and/or (b) correlation between the expression of this fetal-like phenotypic characteristic and either cell morphology or growth dynamics.

The lamina propria of attached gingiva is divided into papillary and reticular layers, the papillary body forming numerous finger-like projections (papillae) that interdigitate with overlying epithelial rete pegs (Fig. 2A,B). The appearance and 3-D association of papillae and epithelial rete pegs are more apparent in scanning electron micrographs of separated epithelial and connective tissue components (Fig. 2C,D). In such preparations, most papillae stood erect with blunt tips, which were occasionally bifid. The papillae were slightly tapered and measured on average 60±21 μm in diameter and 200±49 μm in length, these dimensions being similar to those reported by Klein-Szanto and Schroeder (1977). The basal surface of the gingival epithelium contained rows of papillary openings with relatively flat interpapillary areas.

The erect nature of the papillae allowed their dissection from the underlying reticular tissue, usually in groups of two to three. Primary cultures of PAP and RET fibroblasts were obtained as outgrowths from small fragments of the respective tissues embedded in 0.5 ml collagen gels. Cellular outgrowth from papillae markedly preceded that from reticular tissues (Fig. 3). Papillae obtained from all of the gingival biopsies examined in this study always yielded visible cellular outgrowth within 48 hours, with the cultures requiring subcultivation within 10-14 days. In contrast, cellular outgrowth from the explanted pieces of RET tissue was never observed prior to the 7th day of incubation, this yielding a sufficient number of cells for subcultivation after a further 2-3 weeks. Fibroblast outgrowth occurred from a total of 92% PAP explants compared to only 36% similarly sized RET explants.

When cultured either on plastic dishes or on the surface of collagen gels, PAP fibroblasts displayed a relatively small (81±21 μm, n=50), spindle-shaped morphology (Fig. 4A,C) compared to the considerably larger (210±56 μm, n=50) and more spread appearance of the RET cells (Fig. 4B,D). Interestingly, PAP and RET cells were morphologically indistinguishable when cultured within a 3-D collagen matrix, with both cell types adopting a stellate appearance of similar size (not shown).

Three pairs of PAP and RET fibroblasts, as well as the corresponding unselected explant-derived (NOM) cells obtained from the same gingival biopsies, were studied in further detail. In the first instance, the migration of these cells was assessed at both high and low cell densities and these data were used to calculate the CDMI. The results obtained with these cells are presented in Table 3. All of the PAP fibroblast lines consistently expressed fetal-like CDMI values, whilst the RET cell lines were equally consistent in expressing adult-like CDMI values. The unselected NOM fibroblasts resembled the PAP cells in that they expressed a fetal-like migratory phenotype (consistent with the data presented in Table 2).

Growth curves obtained with an unselected gingival fibroblast line (NOM339) and the corresponding PAP and RET fibroblast subpopulations are presented in Fig. 5; related data summarizing the saturation cell densities achieved by three lines of NOM, PAP and RET fibroblasts (as well as representative fetal and adult skin fibroblast lines) are presented in Table 4. In these experiments, cells were grown on plastic dishes and within 3-D collagen gels in the presence of 2.5% (low) and 15% (high) fetal calf serum. The data indicate that PAP fibroblasts achieved higher saturation cell densities compared to their matched RET cells under all culture conditions. The growth characteristics of the unselected NOM fibroblasts were indistinguishable from that of the PAP cells, thereby suggesting that PAP cells were the principal fibroblast subpopulation in the NOM lines. No consistent differences were observed between the three groups of PAP and RET cells with regard to the effect of substratum (plastic v collagen) on cell proliferation.

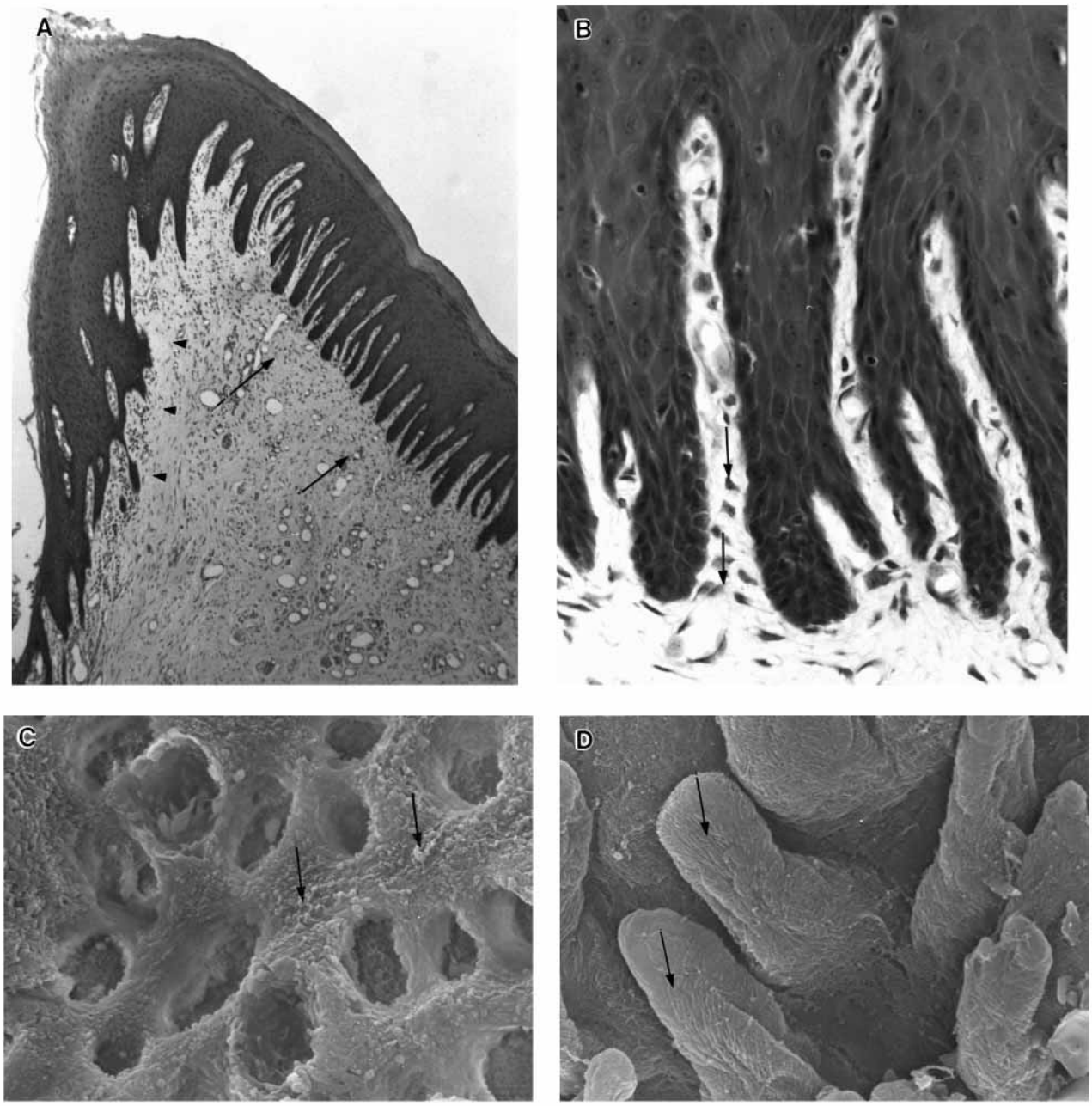

Fig. 2. Human gingival mucosa. (A) Low magnification of haematoxylin and eosin-stained gingival tissue section showing prominent papillae in outer gingival mucosa (arrows) compared to the sulcular attachment (arrowheads). ×25. (B) Higher magnification of gingival papillae showing numerous fibroblasts (arrows). ×100. (C and D) Scanning electron micrographs of trypsin-split outer gingival mucosa showing the arrangement of the epithelial undersurface (C) into rete ridges (arrows) and the opposing connective tissue lamina propria (D) with numerous papillae (arrows). ×225.

Production of MSF by PAP and RET fibroblasts

In order to determine the correlation between expression of a fetal-like CDMI and production of MSF, conditioned media (CM) produced by the three paired lines of PAP and RET fibroblasts were as assayed for the presence of migration-stimulating activity both prior to (i.e. neat) and following fractionation according to the protocol previously described for the purification of MSF (Schor et al., 1988a; Grey et al., 1989). As can be seen in Fig. 6A, MSF activity is present in fractions 11 and 12 of fractionated PAP fibroblast CM, this activity profile being identical to that displayed by a fetal skin fibroblast line (Fig. 6C). In contrast, no detectable MSF activity was apparent in either fractionated RET (Fig. 6B) or control adult skin (Fig. 6D) fibroblast CM. Similar data relating to MSF production by the three paired PAP and RET fibroblast lines are summarized in Table 3. Detectable migration-stimulating activity attributable to MSF was present in all of the neat and fractionated NOM and PAP CM samples and absent from all of the corresponding RET samples. The presence of MSF in the neat CM

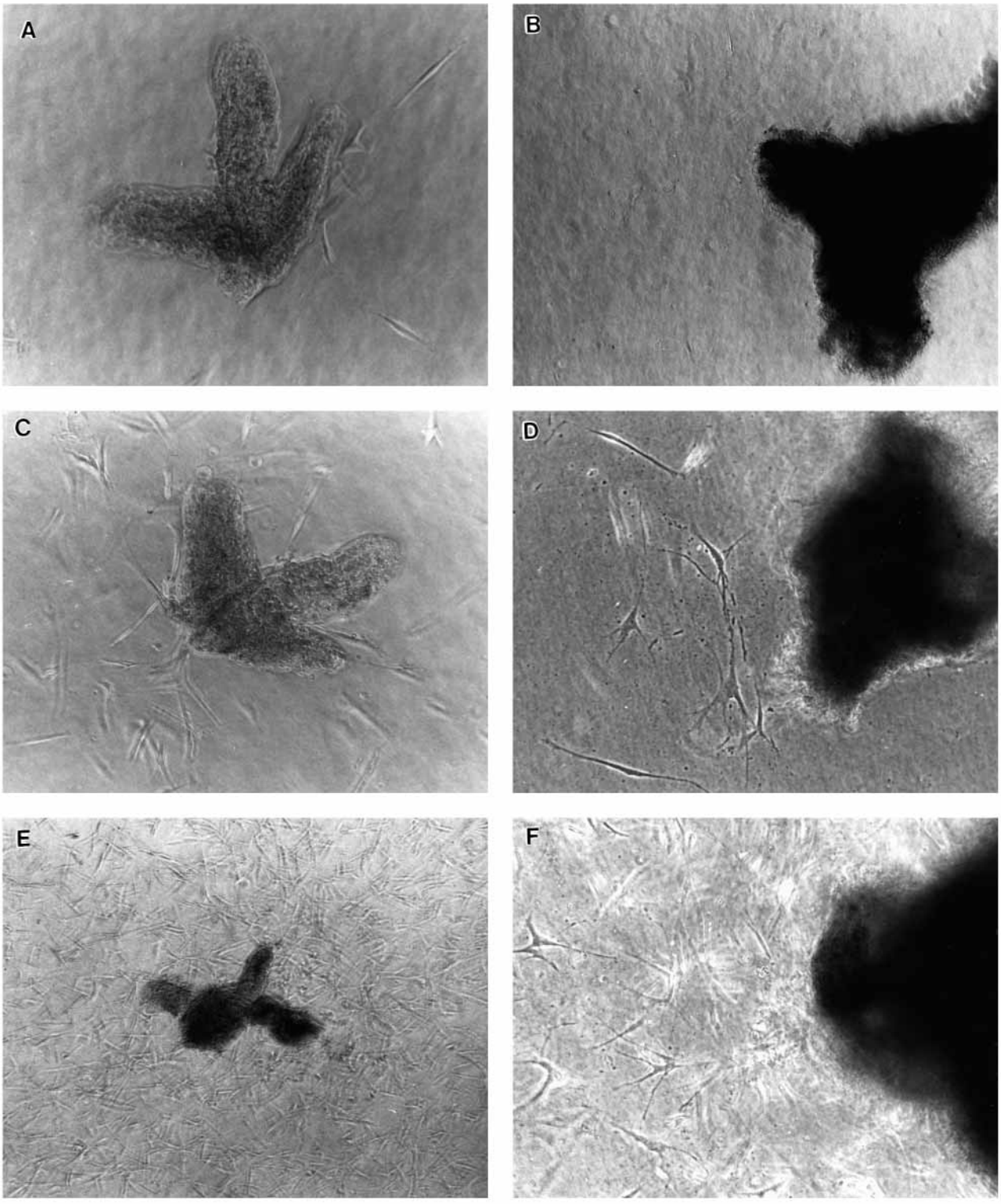

Fig. 3. Fibroblast outgrowth from explanted papillary tips and reticular tissue from microdissected gingival biopsy. Separated gingival mucosa was microdissected into papillary tips and regions of deeper reticular tissue. These were explanted onto 3-D collagen gel substrata to allow fibroblast outgrowth. Photomicrographs of the explanted papillary tissue were taken after 2 days (A), 7 days (C) and 12 days (E). Fibroblast outgrowth from the explanted reticular tissue was consistently slower, as indicated by micrographs taken at 2 days (B), 14 days (D) and 24 days (F). A-D and F, ×136; E, ×86.

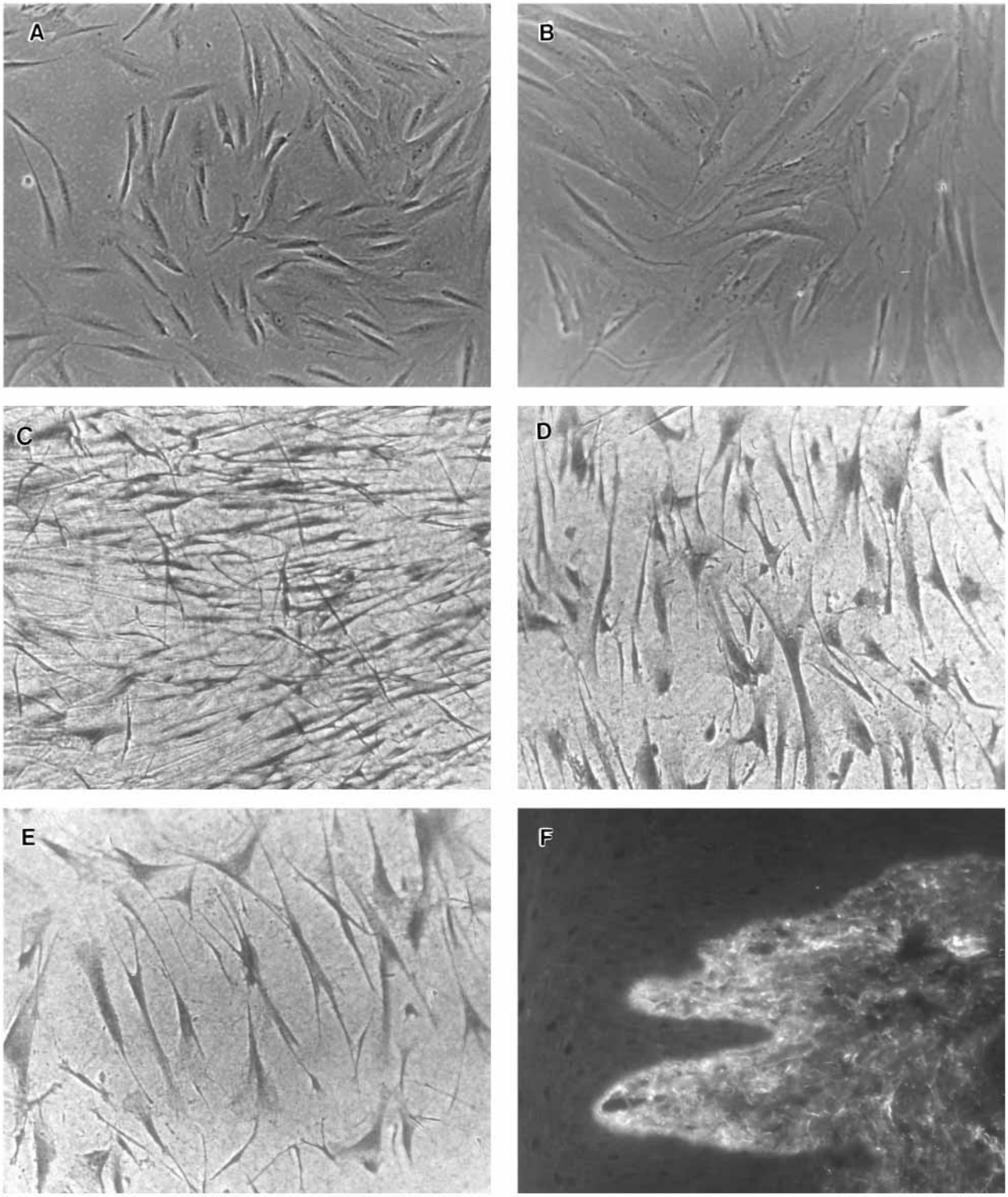

Fig. 4. PAP and RET gingival fibroblasts in cell culture and the immunolocalization of MSF in gingival tissue. Passage 3 PAP fibroblasts cultured on plastic (A) and collagen substrata (C) are significantly smaller than similarly cultured RET cells (B, plastic; and D, collagen). PAP fibroblasts at passage 10, growing on a collagen substratum (E) are larger and indistinguishable from their RET counterparts (A-E, ×136). (F) Immunofluorescence preparation of outer gingival mucosa using anti-MSF antibody showing more intense staining in the papillary region compared to the reticular layer. ×200.

Table 3. CDMI values and MSF production by PAP and RET fibroblasts

Cell line	%LD	%HD	CDMI	Class	Stimul (neat CM) −ab	Stimul (neat CM) +ab	Stimul (fract CM) −ab	Stimul (fract CM) +ab
Gingiva								
PAP317	16.8	8.0	0.32	F	**2.2**	1.0	**1.9**	1.2
RET317	12.3	3.2	0.58	A	1.3	1.4	1.0	1.0
NOM317	14.3	9.3	0.17	F	**2.6**	0.9	**2.3**	1.3
PAP339	11.0	5.3	0.31	F	**2.3**	1.1	**2.2**	1.3
RET339	10.1	3.7	0.44	A	1.1	1.2	1.4	1.4
NOM339	12.1	6.2	0.29	F	**2.7**	0.9	**2.9**	1.2
PAP438	8.0	4.8	0.23	F	**1.9**	0.9	**2.0**	1.2
RET438	14.7	4.3	0.53	A	1.1	0.9	0.7	1.2
NOM438	14.2	7.1	0.30	F	**2.5**	1.4	**1.9**	1.0
Adult skin								
SK156	14.9	3.9	0.58	A	0.9	0.9	0.7	0.9
SK271	10.2	2.2	0.67	A	1.5	1.2	1.1	1.3
Fetal skin								
F110a	12.7	8.8	0.16	F	**2.7**	1.4	**2.2**	1.0
F111	15.2	7.5	0.31	F	**2.0**	1.0	**1.9**	0.9

Data are presented concerning the migration of nonselected explant cultures of normal oral mucosal (NOM) fibroblasts, and PAP and RET fibroblasts derived from the same gingival tissue. Cells were plated at low density (%LD) and high density (%HD), the CDMI calculated on the basis of these data and the various lines classified (Class) as expressing either an adult-like (A) or fetal-like (F) migratory phenotype. The presence of migration stimulating activity in both neat CM and fraction 12 of fractionated CM was assessed as described in Materials and Methods; these data are expressed as the relative stimulation of migration (Stimul) of a target adult fibroblast line in the presence (+ab) and absence (–ab) of 1 μg/ml anti-MSF polyclonal antibody continuously during the 4 day incubation period; values greater than 1.8 (shown in bold) are considered as indicative of significant migration stimulating activity (Picardo et al., 1991). The anti-MSF antibody was purified by sequential passage through a Protein A and two fibronectin affinity columns; this antibody did not recognise either fibronectin or its isolated gelatin-binding domain in western blots or ELISA.

Table 4. The effects of serum concentration and substratum on saturation cell density

	Cell number ($\times 10^{-4}$)			
	On plastic		In collagen	
Cell line	2.5% Serum	15% Serum	2.5% Serum	15% Serum
Gingiva				
PAP317	7.1	23.1	6.8	18.6
RET317	4.5	10.7	4.1	13.1
PAP339	35.9	81.8	63.2	254.0
RET339	11.7	27.0	15.2	31.4
PAP438	10.4	23.6	9.6	28.5
RET438	6.1	10.6	6.3	11.3
Adult skin				
SK156	9.1	15.1	10.7	15.7
SK271	6.5	18.6	14.2	25.7
Fetal skin				
F110a	27.7	138.4	77.8	160.0
F111	70.9	233.3	107.7	322.1

Cells were seeded at a density 2×10^4 cells/culture on either 35 mm plastic tissue culture dishes or within 3-D collagen gels in the presence of MEM containing either 2.5 or 15% fetal calf serum. Growth medium was changed every three days and the cell number in duplicate cultures determined at three-day intervals. Saturating cell densities (i.e. not altered in two consecutive measurements) were achieved after 10-21 days, depending on culture conditions. The presented data represent the means of two independent experiments in which the s.d. was less than 10% of the indicated means.

and active CM fractions was confirmed by the apparent neutralization of its biological activity with an affinity-purified anti-MSF antibody (Schor et al., 1993). These observations indicate a tight correlation between expression of a fetal-like CDMI and production of MSF (Fig. 1). This correlation is further supported by data presented in Table 5, indicating that incubation of NOM, PAP and fetal skin fibroblasts with the anti-MSF antibody resulted in an increase in the CDMI to values falling within the adult range; similar incubation of RET and adult skin cells with the antibody had no significant effect upon cell migration.

The anatomical location of antibody-reactive MSF in sections of attached gingival tissue is shown in Fig. 4F. Tissue sections were exposed to affinity-purified anti-MSF antibody and bound antibody visualized by reaction with an anti-rabbit FITC-labelled secondary antibody. A diffuse staining of the papillary regions of the lamina propria was obtained, with both the underlying reticular regions and overlying epithelium being less immunoreactive. These observations support the in vitro data indicating a preferential localization of MSF-producing fibroblasts in the papillary regions of the lamina propria. Control preparations exposed to nonimmune serum were devoid of staining (not shown).

The effects of in vitro passage on the migratory phenotype of PAP fibroblasts

We have previously reported that fetal skin fibroblasts ‘aged’ in vitro undergo a spontaneous transition characterized by the adoption of an adult-like migratory phenotype (Schor et al., 1985a) and cessation of MSF production (Schor et al., 1988a). Data are presented in Table 6 indicating that PAP fibroblasts underwent a similar spontaneous transition in CDMI. Subcultured PAP cells displayed CDMI values falling within the fetal range during the initial period of their in vitro lifespan, but had adopted an adult-like migratory phenotype when examined at passage 10; this change in migratory phenotype was associated with a corresponding cessation in MSF production. Interest-

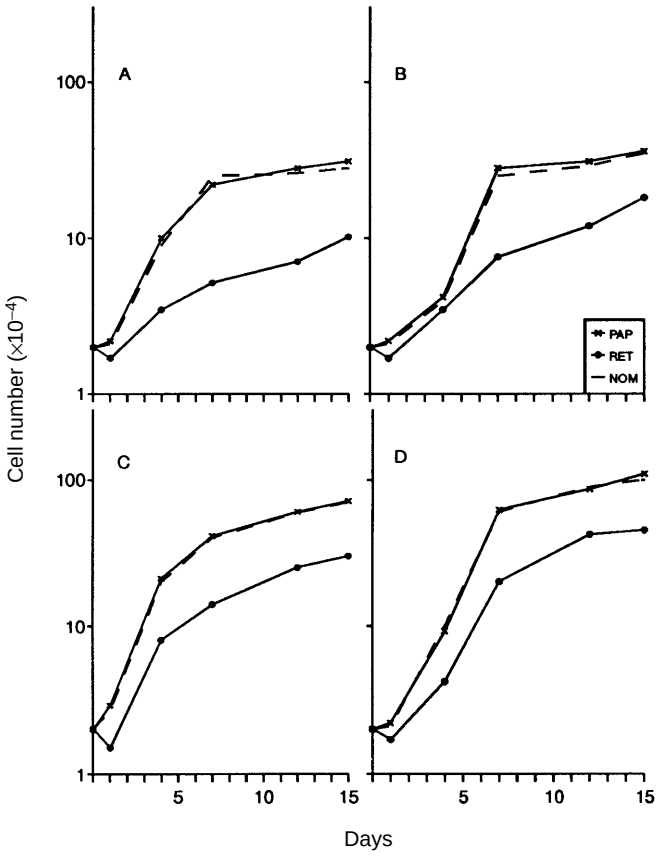

Fig. 5. The proliferation of NOM, PAP and RET gingival fibroblasts. NOM339 fibroblasts and the corresponding PAP and RET cells derived from the same tissue were plated at 2×10^4 cells per culture on 35 mm plastic tissue culture dishes (A,C) and within 3-D collagen gels (B,D) in the presence of 2.5% (A,B) and 15% (C,D) fetal calf serum. Cell numbers were determined at intervals thereafter as described in Materials and Methods.

ingly, the aged PAP fibroblasts also adopted a spread morphology essentially indistinguishable from that of the RET cells (Fig. 4e). Subcultured RET fibroblasts consistently displayed an adult migratory phenotype during the entire duration of their (limited) in vitro lifespan.

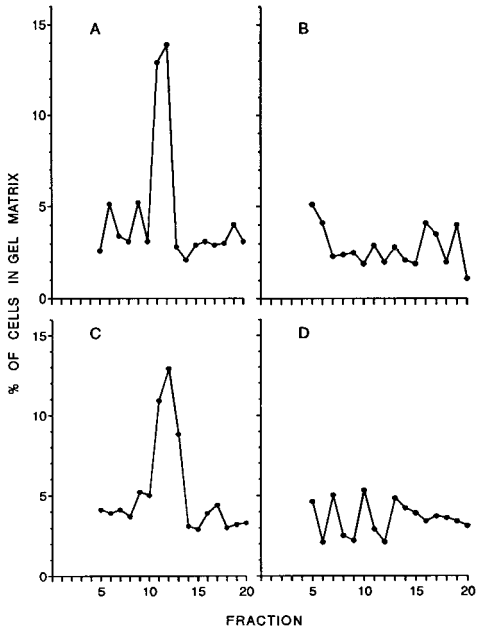

Fig. 6. The presence of migration stimulating activity in fractionated fibroblast conditioned medium. Conditioned medium produced by PAP339 fibroblasts (A), RET339 fibroblasts (B), F110a fetal skin fibroblasts (C) and SK339 adult skin fibroblasts (D) were fractionated by gel filtration chromatography and the presence of migration stimulating activity in the resultant 1 ml fractions was assayed as described in Materials and Methods.

DISCUSSION

Data presented in this paper indicate that: (a) fibroblasts obtained from total explant cultures of gingiva differ from similarly derived (donor-matched) dermal fibroblasts in terms of their expression of a fetal-like migratory phenotype and production of MSF; (b) only fibroblasts derived from the gingival papillae displayed these fetal-like phenotypic characteristics, whilst gingival fibroblasts obtained from the deeper reticular region resembled skin cells; (c) PAP fibroblasts also differed from RET cells in terms of their smaller (more fetal-like) morphology and relatively enhanced proliferative potential; and (d) cultured PAP fibroblasts spontaneously adopted a RET phenotype during the course of cell ageing in vitro, which is characterized by a cessation in MSF production and the

Table 5. The effects of the anti-MSF antibody on fibroblast migratory phenotype

	–ab				+ab			
Cell line	%LD	%HD	CDMI	Class	%LD	%HD	CDMI	Class
PAP317	18.9	11.2	0.28	F	17.9	4.9	0.56	A
RET317	14.6	4.2	0.54	A	16.8	4.2	0.60	A
NOM317	14.5	9.9	0.17	F	12.2	4.2	0.46	A
Adult fibroblasts								
SK156	15.0	4.2	0.53	A	11.3	4.1	0.44	A
Fetal fibroblasts								
F110a	18.9	8.9	0.33	F	16.1	4.9	0.52	A

Cells were plated at low density (%LD) and high density (%HD) in the presence (+ab) and absence (–ab) of affinity-purified anti-MSF polyclonal antibody; the CDMI was calculated on the basis of these data and the various lines classified (Class) as expressing either an adult-like (A) or fetal-like (F) migratory phenotype.

Table 6. The effect of in vitro passage number on the migratory phenotype of gingival fibroblasts

Cell line	Passage	%LD	%HD	CDMI	Class	Stimul
PAP439	3	10.5	6.1	0.24	F	**2.9**
	5	8.4	5.4	0.19	F	**2.1**
	10	10.8	3.3	0.51	A	1.6
	11	13.4	3.1	0.64	A	1.4
RET439	3	8.3	2.9	0.46	A	1.2
	5	7.7	1.9	0.61	A	1.3

The CDMI expressed by both PAP- and RET439 gingival fibroblasts was determined using stock cells of different passage levels. The PAP cells became senescent at passage 12, whilst the RET cells did so at passage 6. CM produced by these cell lines was fractionated and assayed for MSF activity; these data are expressed as the relative stimulation of migration (Stimul) of a target adult fibroblast line, with values greater than 1.8 (shown in bold) being considered as indicative of significant migration stimulating activity.

adoption of a spread morphology. This PAP-to-RET transition is consistent with our previous observations (Schor et al., 1985a, 1988b) that continuously passaged populations of fetal skin fibroblasts undergo a spontaneous transition to an adult-like migratory phenotype, with concurrent cessation in MSF production, after completing 50-55 population doublings (i.e. approximately 75% of their lifespan in vitro).

Our previously published data suggest that MSF-producing fibroblasts are not a major cell population in healthy adult skin. Indeed, prior to this study, the presence of such fetal-like cells in the healthy adult has only been documented in breast intralobular stroma (Schor et al., 1992).

The preferential localization of MSF-producing (fetal-like) fibroblasts in the gingival papillae is identical to the distribution of tenascin in this tissue (Sloan et al., 1989). This co-distribution pattern is of particular interest as tenascin is a common macromolecular constituent of fetal connective tissue matrices (Chiquet-Ehrismann et al., 1986), where it has been suggested to function in the mediation of epithelial-mesenchymal interactions; interestingly, tenascin is re-expressed in tumour-associated stroma and healing wounds in the adult (Chiquet-Ehrismann et al., 1986; Mackie et al., 1988; Lightner et al., 1990). Immunolocalization of other matrix macromolecules in gingival lamina propria has revealed further differences between the papillary and reticular layers. For example, type I collagen is present in thick bundles throughout reticular tissue, but is more sparsely dispersed in the papillae, where type III collagen tends to predominate in short, thin fibrils (Chavier et al., 1981, 1984, 1985). Interestingly, the relative abundance of type III collagen is also greater in fetal compared to adult skin. In addition, type VI collagen, which forms a microfibriller, almost amorphous pattern throughout the gingival connective tissue, is notably more pronounced in the papillae (Becker et al., 1986). Taken together, these findings suggest that PAP fibroblasts in vivo are also fetal-like with respect to their pattern of matrix synthesis, whereas RET cells are adult-like with respect to these various parameters.

The dermal component of skin resembles gingiva in that it also consists of an upper papillary layer containing a relatively loose network of collagen fibrils and a lower reticular layer containing dense arrays of thicker bundles of collagen fibres. Unfortunately, the lack of prominent connective tissue papillae in forearm dermis has precluded our using the same microdissection technique to obtain distinct subpopulations of papillary and reticular skin fibroblasts; the absence of detectable MSF-secreting fibroblasts in the unselected skin fibroblast cultures does, however, suggest that they are not a major dermal cell population in the healthy adult. Dermal papillary and reticular fibroblasts have been isolated using other techniques and these have been shown to differ with respect to various phenotypic characteristics, including proliferative potential, morphology and ability to contract a collagen lattice (Harper and Grove, 1979; Azzarone and Macieira-Coehlo, 1982; Schafer et al., 1985, 1989). Taken together with our results obtained with gingival fibroblasts, these observations suggest that intra-site fibroblast heterogeneity may be a relatively common feature of connective tissues. Several mechanisms may account for the origin of the distinct PAP and RET fibroblast subpopulations. One possibility is that the different phenotypic characteristics displayed by these cells are induced by the interaction of intrinsically identical fibroblasts with different local concentrations of paracrine factors, as, for example, may be produced by the overlying epithelium. In support of this model, Chambon and collaborators (Basset et al., 1991) have reported that fetal fibroblasts produce a novel protease, stromelysin-3, which is not made by their normal adult counterparts; interestingly, fibroblasts staining positively for stromelysin-3 were also detected at the tumour-stromal margin in mammary carcinomas, thereby suggesting that interaction with adjacent tumour cells was required for its induction. The maintenance of distinct phenotypic characteristics by PAP and RET fibroblasts under identical culture conditions in vitro does, however, suggest that this mechanism is rather unlikely to account for the differences we report.

An alternative set of possible models is based upon the view that differentiation and/or lineage relationships exist between the different fibroblast subpopulations. In this regard, several previous studies have provided evidence for the differentiation of fibroblasts along a defined pathway, which is recapitulated as a function of cell ageing in vitro (Bayreuther et al., 1988). The particular anatomical location of the PAP and RET cells in the gingiva, taken in conjunction with the phenotypic transition of the PAP cells in vitro, suggests that there may be a downward movement of fibroblasts from the papillary to reticular layers during the course of fibroblast maturation or ageing. A similar model has previously been suggested for pericrypt fibroblasts in the colon (Pascal et al., 1968). In this study, proliferating fibroblasts were identified at the base of the crypts, these lying in close proximity to the proliferating epithelial stem cells. Post-mitotic fibroblasts were demonstrated to migrate up the crypt apparently in tandem with similarly migrating epithelial cells. Collagen deposition was sparse at the crypt base compared to the relatively thick collagen fibre array in the upper regions, thereby suggesting a functional maturation of the fibroblasts as they ascended the crypt wall. A similar migration of fibroblasts is thought to occur in the periodontal ligament, the progenitor cells in this case being located in the mid-zone of the ligament in close proximity to blood vessels with daughter cells migrating towards bone and cementum, where they ultimately die (McCulloch et al., 1989). More recently, McCulloch and Knowles (1991) presented data indicating the presence of three subpopulations of fibroblasts in hamster gingiva with distinct proliferative potential; these included a population of cells that cycled actively both in vitro and in vivo, a second population

of cells that did not divide in vivo, but showed limited proliferative potential in vitro, and a third population of post-mitotic cells that did not cycle either in vivo or in vitro. The tissue localizations of these different fibroblast populations were not determined, although our data suggest that the cells with limited proliferative potential were most likely derived from reticular tissue. Finally, Pender et al. (1988) have demonstrated the presence of progenitor-like cells (these characterized by their elevated mitotic activity) in the papillary region of rat gingiva.

MSF has been detected in wound fluid and has been postulated to play a role in the normal wound-healing response (Picardo et al., 1992). These data further suggest that the MSF present in wound fluid is not derived from degranulating platelets and may be produced by local fibroblasts. It is not known whether such fibroblasts represent an induced population of previously non-MSF-producing cells or an expanded minority population of constitutively producing cells. Related studies concerned with the mechanism of action of MSF indicated that it stimulates the synthesis of high molecular mass hyaluronic acid by target fibroblasts (Ellis et al., 1992), a finding consistent with the influx of fibroblasts into granulation tissue and the elevation in hyaluronic acid synthesis that accompanies the wound-healing response (DePalma et al., 1989; Mast et al., 1992). In this regard, it is interesting to note that wound healing in the oral mucosa has commonly been reported to be fetal-like in that it occurs rapidly and is not accompanied by either inflammation or scar formation (Hallock, 1985). It is possible that the presence of MSF-producing fibroblasts in the connective tissue papillae may contribute to this beneficial mode of wound healing. Interestingly, fibroblasts in granulation tissue also appear to express a number of other fetal-like characteristics, including the synthesis of fetal isoforms of fibronectin (ffrench-Constant et al., 1989).

We have previously reported that skin fibroblasts obtained from patients with both sporadic and familial breast cancer commonly display a fetal-like migratory phenotype and produce MSF. In this regard, it is of interest to note that the tumour-stroma interface displays a number of similarities to the wound environment, including matrix degradation and remodelling, a fact that prompted Dvorak (1986) to compare tumours to ‘wounds that do not heal’. In this sense, it may be that the mechanisms that both temporally and spatially limit the production of MSF during wound healing may be subverted in cancer patients, thus resulting in the prolonged and systemic persistence of MSF-producing fibroblasts in these individuals. We have further suggested that: (a) the presence of persistent MSF-producing fibroblasts in breast cancer patients directly contributes to disease progression by virtue of their disruption of normal epithelial-mesenchymal interactions (Schor et al., 1987, 1991); and (b) these fetal-like fibroblasts are derived from the clonal expansion of a distinct pre-existent subpopulation of cells (Schor et al., 1993,1994). The data presented in this paper are consistent with the existence of such minority subpopulations of MSF-producing fibroblasts in the healthy adult.

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