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Colonic pericryptal fibroblasts

Differentiation pattern in embryogenesis and phenotypic modulation in epithelial proliferative lesions

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Summary. Experimental evidence has shown that fetal gut mesenchymal cells can modulate epithelial cell differentiation. It is postulated that reciprocal stromal-epithelial interactions in the digestive tract are maintained beyond embryonic life. The mature colonic mucosa contains pericryptal fibroblasts (PCF), a stromal cell type exhibiting smooth muscle morphological features, which are thought to regulate the growth and differentiation of adjacent epithelial cells. Using an antibody directed at α-smooth muscle actin, which is constantly expressed in smooth muscle cells, we performed an immunohistochemical study on human embryonic tissues to assess PCF differentiation during development. PCF expressing α-smooth muscle actin were first detected around the 21st week of gestation, at the bases of the crypts; the number of differentiated PCF increased then progressively, in synchrony with epithelial proliferation, to achieve at birth the characteristic distribution found in adults. We analyzed a series of non-malignant and malignant epithelial proliferative lesions of the adult colon by the same technique. Only sparse immunoreactive PCF were observed in 10/10 pure tubular adenomas, whereas in 11/11 villous adenomas immunoreactive PCF were consistently found bordering proliferative epithelia. Interestingly, 3/5 papillary adenomas, associated with areas of moderate to marked dysplasia, demonstrated foci of multilayered immunoreactive PCF. In 14/14 carcinomas examined, PCF were no longer recognizable; stromal cells expressing variable amounts of α-smooth muscle actin, constituting the desmoplastic reaction, were constantly present. These observations establish immunohistochemically a smooth muscle phenotypic feature of PCF, which is acquired at mid-gestation, and the ability of PCF to proliferate in conjunction with some epithelial neoplasias. These findings might help to clarify the histogenesis of PCF and to improve our understanding of the mesenchymal-epithelial interactions suspected to operate during organogenesis as well as benign and malignant neoplastic conditions.

Key words: Actin isoforms – Myofibroblasts – Desmoplasia – Gut development – Carcinoma of the colon

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Introduction

The use of differentiation markers, such as cytoskeletal proteins, has recently furnished an accurate definition of mesenchymal cell phenotypic heterogeneity which was essentially based on morphological analysis (see for review Skalli and Gabbiani 1988). Immunological probes directed at intermediate filament proteins and actin isoforms have allowed the identification of an array of differentiation patterns amongst stromal cells (Glasser and Julian 1986; Toccanier-Pelte et al. 1987; Skalli et al. 1989a). Moreover, fibroblasts present in granulation tissues and hypertrophic scars (myofibroblasts), which exhibit ultrastructural features typical of smooth muscle (Gabbiani et al. 1972; Seemayer et al. 1981), show a spectrum of smooth muscle differentiation when examined immunohistochemically by means of antibodies directed against cytoskeletal proteins, thus revealing a phenotypic diversity that may account for differing biological behaviour (Skalli et al. 1989a).

Interactions between stromal and epithelial cells are known to govern organogenesis: embry-

onic mesenchymal cells are capable of inducing gut epithelial cells to differentiate (Kedinger et al. 1986) and, conversely, mesenchymal cell differentiation is influenced by epithelial cells (Sakakura et al. 1979). Animal studies have suggested that similar stromal-epithelial interactions may operate beyond embryonic life (Sakakura et al. 1979; Hafen et al. 1987; Lacroix et al. 1984; Kratochwil 1986). Therefore, the analysis of mesenchymal cell differentiation may help in understanding the mechanisms involved in epithelial growth control.

Colonic pericryptal fibroblasts (PCF) belong to a specialized subset of mesenchymal cells, displaying morphological smooth muscle differentiation features associated with migrating and proliferative properties (Kaye et al. 1968). Though their function(s) remain speculative, some authors have postulated that PCF may exert a direct control on epithelial cell differentiation after birth (Hafen et al. 1987). As a continuously self-renewing structure, the colonic mucosa is a preferential target for epithelial growth dysregulations, ranging from hyperplasia to carcinoma. Adenomatous lesions are widely considered as precursors of colonic cancer, but the identification of pre-malignant lesions is still a matter of controversy, particularly in intermediate-size adenomas (Sugarbaker et al. 1985; Nowell 1988). Modulations of stromal cell phenotype in the vicinity of proliferating epithelia have been reported in other tissues (Sappino et al. 1988; Chapponier and Gabbiani 1989), suggesting that their analysis might carry a biological, diagnostic and prognostic significance.

Using an immunological probe directed at α-smooth muscle actin – the actin isoform present in all types of smooth muscle cells (Skalli et al. 1987) – which has been shown to recognize PCF in adult tissues (Widgren et al. 1988), we have studied immunohistochemically the colonic mucosa of human embryos at various stages of development, to clarify the correlation between PCF differentiation and glandular crypt formation. In addition, we have studied by the same technique a series of non-malignant and malignant adult colonic tissues, to assess the expression and the differentiation pattern of PCF in non-malignant and malignant epithelial proliferative conditions.

Material and methods

Colonic mucosae from aborted embryos were collected prospectively, fixed in formalin and embedded in paraffin. Specimens from fetuses devoid of major malformations and without light microscopic abnormalities of the gut mucosa were selected for analysis. They included: 2 samples at 19 weeks of gestation, 3 samples at 21 weeks, 2 samples at 24 weeks, 1 at 26 weeks, 1 at 29 weeks, 2 at 32 weeks, 2 at 34 weeks and 2 at term. Colonic specimens from 3 infants who died of the sudden death syndrome at 1 and 2 months of age were selected from the biopsy collection of our Department.

Routinely processed formalin-fixed and paraffin-embedded colon tissues were selected retrospectively on the basis of conventional histological assessment. Nonmalignant specimens included: 6 samples from colectomies performed for diverticulitis, 6 samples of distal mucosa from colectomies performed for carcinoma, 10 samples of tubular adenomas, 11 samples of villous adenomas without dysplasia and 5 with moderate dysplasia, and 6 samples of hyperplastic mucosa from familial polyposis. Malignant specimens included 14 samples of carcinomas with analysable non-malignant adjacent epithelium.

All specimens were reviewed by one of us (S.W.). α-smooth muscle actin was detected with a monoclonal antibody (anti-α-sm-1) (Skalli et al. 1986) and by means of the avidin-biotin complex (ABC) peroxidase method using formalin-fixed paraffin-embedded material, as previously described (Sappino et al. 1988).

Appropriate controls included the omission of the primary antibody and purified mouse non-immune immunoglobulins at the same concentration as the first antibody.

Photographs were taken with a Zeiss photomicroscope (Zeiss, Oberkochen, FRG) using Kodak Ektachrome 50 colour film (England).

Results

Anti-α-sm-1 detected, as expected, smooth-muscle cells present in the different muscular layers of the colon, in the media of the vessels and pericytes (Skalli et al. 1989b). In all samples analyzed, the intensity of staining in the cells from the muscularis mucosae appeared stronger than in the longitudinal and circular muscle layers. This is probably due to the higher content of α-smooth muscle actin in smooth muscle cells of the muscularis mucosae when compared with those of the muscle layers, which are known to contain a high proportion of γ-smooth muscle actin (Skalli et al. 1987).

At 19th week, the colonic mucosa comprises fully developed muscular layers, including the muscularis mucosae, and tubular pits lined with proliferating immature epithelial cells, separated by stromal axes. At that stage, no immunoreactive PCF were detected, the immunostaining being limited to the muscular layers and to the prolongations of the muscularis mucosae sprouting in-between the crypts, consisting of smooth muscle cells and probably of some pericytes (not shown). At 21 weeks, the first PCF expressing α-smooth muscle actin were detected, in immediate contiguity to the muscularis mucosae, at the bases of the crypts (Fig. 1 A). Between the 21st and 39th weeks, the number of PCF apposed to the basal lamina of epithelial cells, increased progressively from the lower-third up to the midth of the crypts, in syn-

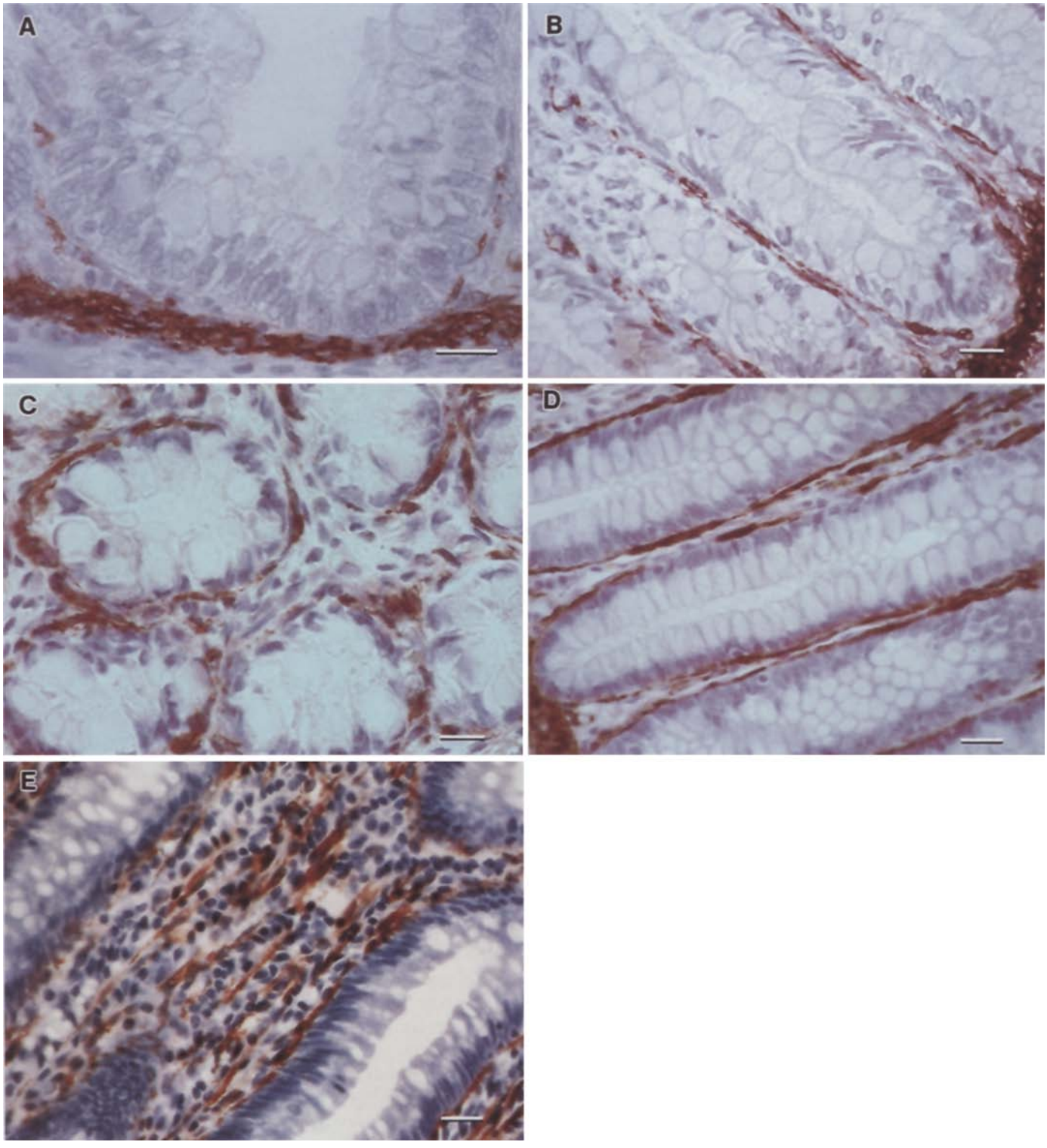

Fig. 1 A–E. Peroxidase staining of paraffin-embedded colonic mucosa from embryonic and adult specimens, 5 μm thick sections, with anti-α-sm-1 (see "Material and methods"). *Bar*: 10 μm. **A** 21 week-old embryo: anti-α-sm-1 decorates the muscularis mucosae and only few individual cells surrounding the bases of the crypts. **B** 24 week-old embryo: immunoreactive PCF are arranged in sheaths, lining the epithelial cells, up to the midth of the glands. **C** 39 week-old embryo: transverse section of glandular structures showing a layer of positively labelled PCF surrounding epithelial cells. **D** Normal adult colonic mucosa: cryptic glands are lined by immunoreactive PCF and a few labelled cells are found in the sub-luminal collagen table. **E** Hyperplastic mucosa from familial polyposis: PCF are intensively labelled and numerous positively stained cells are recognized in the sub-epithelial space, distant from the epithelial basement membranes

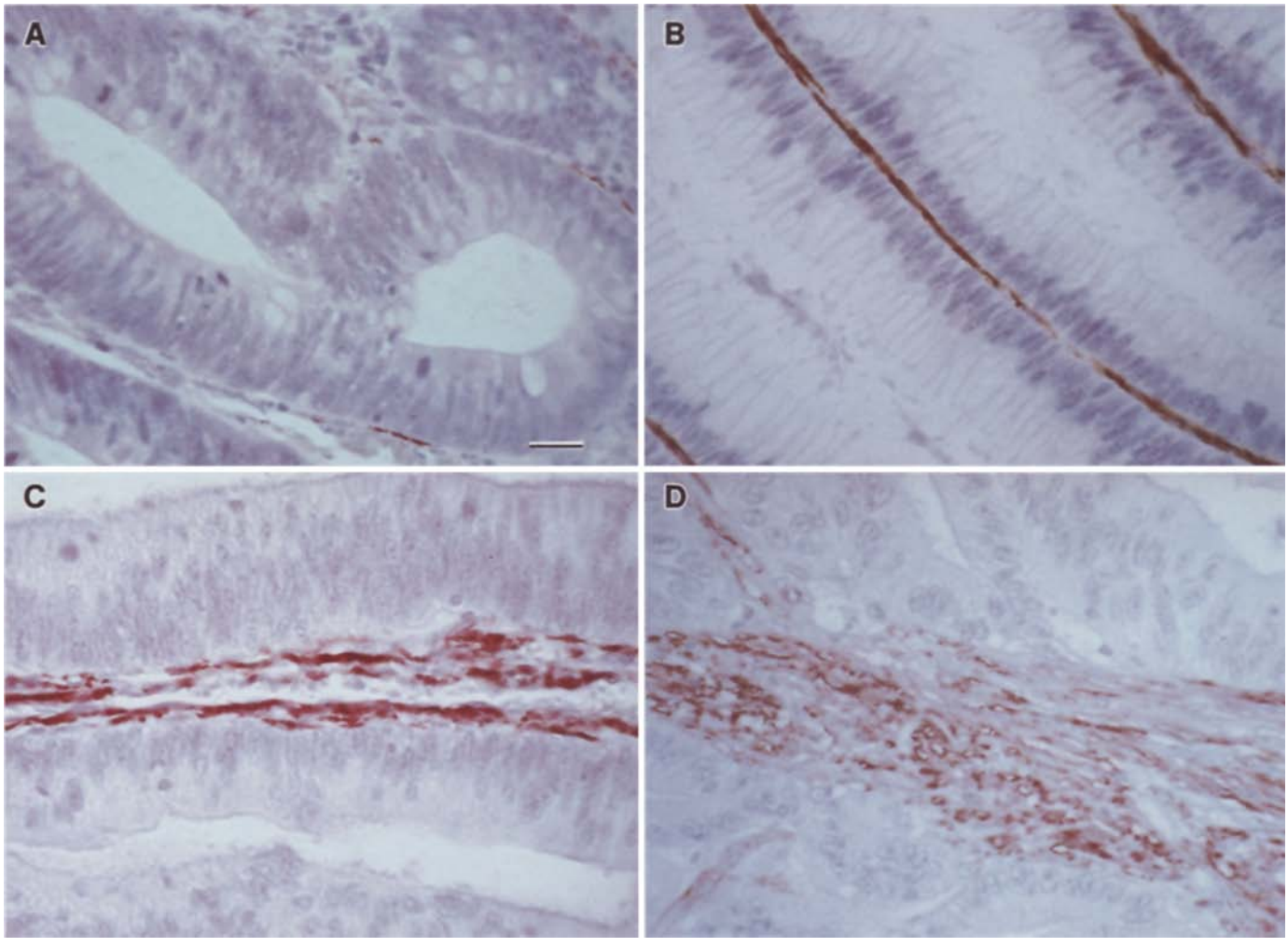

Fig. 2A–D. Peroxidase staining of paraffin-embedded colonic mucosa from adult specimens, 5 μm thick sections, with anti-α-sm-1 (see "Material and methods"). *Bar*: 10 μm. **A** Tubular adenoma: only few immunoreactive PCF can be recognized. **B** Villous adenoma: Immunoreactive PCF follow proliferating glands as a continuous layer apposed to the basement membrane of epithelial cells. **C** Villous adenoma with moderate dysplasia: PCF decorated by anti-α-sm-1 are arranged in multiple layers, in regard of the typical "picket-fence" adenomatous epithelium. **D** Carcinoma: Few immunoreactive cells dysplay a pericryptic distribution, whereas stromal cells constituting the desmoplastic reaction show a variable intensity of staining with anti-α-sm-1

chrony with the proliferation and maturation of epithelial cells. From the 21st week until birth, all recognizable PCF displayed anti-α-sm1 immunoreactivity (Fig. 1 B and 1 C). At term, the characteristic distribution of PCF lining the lower 2 thirds of the glandular crypts was achieved and, at that stage, all identifiable PCF were again found to express α-smooth muscle actin (Fig. 1 C).

The number and the distribution of immunoreactive PCF was constant in the various specimens of colonic mucosa considered to be microscopically devoid of epithelial abnormalities (Fig. 1 D). This pattern was similar to the one seen in fetuses at term, though some positive cells appeared also under the free surface of the epithelium. In two cases, various segments of the colon were analyzed: we observed an identical pattern of PCF expression in the ascending, transverse and sigmoid portions.

In the 6 samples from familial polyposis displaying simple hyperplasia of the mucosa, the number and the intensity of staining of immunoreactive PCF was increased when compared to specimens of non-proliferating epithelia (Fig. 1 E). In 10/10 cases of pure tubular adenomas, the majority of adenomatous glands were devoid of a PCF sheath. Only a restricted number of labelled cells were therefore observed in close association with proliferative epithelia (Fig. 2 A).

In contrast, in the 11 villous adenomas, a persistent sheath of α-smooth muscle actin positive cells was found, which bordered proliferative epi-

thelia up to the extremities of finger-like villi (Fig. 2B). In 3/5 villous adenomas with moderate to marked dysplasia, in addition to the persistent sheath of PCF, foci of immunoreactive cells were seen, which were arranged in multiple layers in the immediate vicinity of dysplastic areas, suggesting they had proliferated in these locations (Fig. 2C).

In the 14 cases of carcinoma examined, immunoreactive PCF were not recognizable around carcinomatous strands; however, an important amount of stromal cells decorated by anti-α-sm-1 and constituting the desmoplastic reaction was always noted (Fig. 2D). The hyperplastic mucosa adjacent to the carcinomas displayed a persistent layer of immunoreactive PCF, whereas dysplastic glands showed foci of proliferating positive cells detached from the epithelial compartment, similar to the pattern observed in villous adenomas with dysplasia. At the periphery of the tumours, some carcinomatous glands were noticed to be surrounded by multiple layers of positively stained cells, whereas in the central parts of the tumours, the typical desmoplastic reaction displayed rows of stromal cells with a highly heterogeneous pattern of immunoreactivity. However, the intensity of anti-α-sm-1 staining appeared less pronounced when compared to the immunoreactivity previously observed in the desmoplastic cells accompanying breast carcinomas (Sappino et al. 1988).

Discussion

The importance of mesenchymal-epithelial interactions in the organogenesis of the digestive tract has been particularly well illustrated by in vitro and grafting experiments, studying the differentiation of epithelial progenitor cells (Kedinger et al. 1986; Hafen et al. 1987). Evidence has accumulated suggesting that such regulatory mechanisms are perpetuated beyond embryonic life and may be disrupted during pathological conditions (Sakakura et al. 1979; Hafen et al. 1987; Kratochwil 1986). Indeed, PCF belong to a specialized subset of stromal cells thought to exert influence on the differentiation program of adjacent epithelial cells before and after birth. They express smooth muscle morphological features, which has led to the suggestion they might also exert functions similar to those of myoepithelial cells (Lacroix et al. 1984; Kaye et al. 1968).

The availability of an immunological probe directed at the NH_2-terminal decapeptide of α-smooth muscle actin offers the opportunity to assess smooth muscle differentiation (Skalli et al. 1986) and provides a way to compare phenotypic modulations in stromal cells during development and pathological processes. Up to now, α-smooth muscle actin has been localized in normal smooth muscle cells (Skalli et al. 1986), pericytes (Skalli et al. 1989b), myoepithelial cells and some stromal cells (Skalli et al. 1986); it appears functionally related to the contractile activities of smooth mucle cells (Kocher and Gabbiani 1987). We confirm here immunohistochemically the expression of a smooth muscle differentiation feature by PCF and demonstrate that this feature is acquired in utero: the first stromal cells with a pericryptic distribution, expressing α-smooth muscle actin, are identifiable around the 21st week of gestation, at the lower third of the crypts. From then until birth, the number of immunoreactive PCF increases progressively. These observations suggest that differentiated PCF are endowed with migrating and proliferating capacities during embryonic life. The data we obtained in the analysis of epithelial proliferative states show that such properties may be conserved in the adult colon and corroborate previous reports based on morphological assessment and thymidine labelling (Kaye et al. 1968 and 1971). However, if hyperplastic epithelia are consistently surrounded by a sheath of PCF expressing α-smooth muscle actin, adenomatous lesions differ in their PCF content according to their histological type: rare differentiated PCF were found in association with tubular adenomas, whereas villous adenomas displayed a persistent layer of immunoreactive cells overlaying the neoplastic epithelium. This finding apparently contradicts morphological studies describing fibroblasts from adenomatous polyps (Kaye et al. 1971; Ohtani and Sasano 1983) and may be attributed to the differences in the sampling of specimens, as well to the use of a probe detecting smooth muscle differentiation. In 3 of 5 villous adenomas, immunoreactive stromal cells were arranged in multiple layers, suggesting a proliferation of differentiated stromal cells, reminiscent of what was previously observed in breast tissues (Sappino et al. 1988). Similarly, we noticed an increased expression of differentiated PCF in all 6 specimens of familial polyposis analyzed; interestingly, these cells were abundant in the proximity of hyperplastic epithelia devoid of adenoma. In carcinomas, a variable proportion of the mesenchymal cells constituting the desmoplastic reaction were found to express α-smooth muscle actin, without regard to a periepithelial organization. As previously demonstrated, the stromal cells of desmoplasia were recognized by anti-α-sm-1 (Skalli et al. 1986; Sappino et al. 1988), though the heterogene-

ity of staining was more pronounced in colonic carcinomas than in breast or lung carcinomas.

The molecular mechanisms underlying reciprocal epithelial-stromal interactions in the embryonic life remain undetermined. Our findings do not offer any definite explanation, but raise questions of potential biological and diagnostic significance: they point towards the expression of a stromal cell phenotype shared by the developing epithelium and some neoplasms, that might result from the production of similar signals by growing epithelia. During pathological conditions affecting breast tissues, stromal cells with a smooth muscle differentiated phenotype are found (Sappino et al. 1988). In epithelial proliferations of the mammary gland, various growth factors known to be synthezised by breast epithelial cell lines have been proposed as potential candidates that may be held responsible for stromal cell phenotypic modulations (Peres et al. 1987), though this hypothesis has yet to be confirmed, in particular during development. The inductive properties exerted by epithelial cells on their stromal environment could also be mediated via the production of extra-cellular matrix components, known to influence cell differentiation (Quaroni et al. 1978; Martinez-Hernandez 1988; Inaguma et al. 1988). In addition, our data suggest a histogenetic link between PCF and desmoplastic cells. However, the spectrum of smooth muscle differentiation in mesenchymal cells might be larger than previously suspected (Skalli and Gabbiani 1988; Skalli et al. 1989a) and the shared expression of α-smooth muscle actin by PCF and desmoplastic fibroblasts does not necessarily implicate a common origin; colonic PCF represent a cell type thought to exert specific growth regulations on epithelial cells, as illustrated by their ability to support intestinal endodermal and crypt cell differentiation (Lacroix et al. 1984; Richman and Bodmer 1988) and might therefore still differ from the immunoreactive stromal cells found in lymphoid and breast tissues (Toccanier-Pelte et al. 1987; Sappino et al. 1988).

For the clinician, the determination of pre-neoplastic lesions in the colon constitutes a priority, but the identification of individuals at risk remains controversial, despite the improvements achieved in diagnostic and screening procedures (Bailar and Smith 1986). Evidence has accumulated implying adenomas as precusors of malignant lesions: although numerous investigations have defined functional and genetic alterations present in adenomas and carcinomas (Markowitz et al. 1986; Ponz de Leon et al. 1988; Vogelstein et al. 1988), the precocious detection of lesions susceptible to lead to malignant transformation is still a major challenge. Furthermore, data available on colorectal-tumour development mainly concern the identification of epithelial abnormalities, limited attention has been paid to the modulations affecting the stromal compartment (Kaye et al. 1971; Ohtani and Sasano 1983). As in the breast, our analysis indicates a possible correlation between some neoplasms and the presence of stromal cells with smooth-muscle differentiation features. In consequence, the determination of stromal cell phenotype might help in evaluating lesions with a propensity for malignant transformation. Indeed, immuno-reactive PCF were rarely detected in tubular adenomas, which are generally considered as carrying a minor risk of transformation (Sugarbaker et al. 1985), whereas proliferating immuno-reactive cells were seen in hyperplastic epithelia from familial polyposis specimens and in villous adenomas, which are generally viewed as precursors of carcinomas (Sugarbaker et al. 1985).

Further investigations are clearly needed to unravel the mechanisms underlying the reciprocal mesenchymal-epithelial interactions governing organogenesis and to integrate the differentiation program of PCF in the recently recognized spectrum of mesenchymal phenotypes. Our observations are compatible with the perpetuation of such intercellular processes during pathological conditions and might help in identifying regulatory phenomena shared by embryonic and adult colonic epithelia.

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