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The myofibroblast markers α-SM actin and β-actin are differentially expressed in 2 and 3-D culture models of fibrotic and normal skin

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Abstract

To characterize the differences between fibrotic myofibroblasts and normal fibroblasts, we studied two differentiation markers: α-smooth muscle (SM) actin, a specific marker of myofibroblast differentiation, and β-actin, which is overexpressed in the fibrotic tissue. Experiments were performed on fibroblasts isolated from normal pig skin and on subcutaneous myofibroblasts isolated from pig radiation-induced fibrosis. Three culture models were used: cells in monolayers, equivalent dermis, consisting of fibroblasts embedded into a matrix composed of type I collagen, and *in vitro* reconstituted skin, in which the matrix and containing life fibroblasts were overlaid with keratinocytes. Samples were studied using immunofluorescence and western-blotting. In monolayers cultures, both fibrosis and normal cells expressed α-SM actin. Furthermore, similar amounts of β-actin protein were found. In these conditions, the resulting alterations in the phenotypes of cells made comparison of cultured fibrotic and normal cells irrelevant. Under the two 3-D culture models, normal fibroblasts no longer expressed α-SM actin. They expressed β-actin at the basal level. Moreover, the fibrotic myofibroblasts in both 3-D models retained their differentiation features, expressing α-SM actin and overexpressing β-actin. We found that this normalization was mainly related to the genomic programmation acquired by the cells in the tissue. Cellular motility and microenvironment were also involved, whereas cellular proliferation was not a major factor. Consequently, both three-dimensional models allowed the study of radiation-induced fibrosis *in vitro*, provided good extrapolations to *in vivo* conditions and avoided certain of culture artefacts.

Introduction

A main objective of *in vitro* cell culture is that normal and pathological cells retain the same differentiation features as *in vivo*. To attain this objective, various culture techniques have been developed from the simple monolayer cultures to the complex 3-D models, especially for the culture of skin cells.

The synthesis of reconstructed dermis for burn treatment began in 1979, when Bell *et al.* (1979) demonstrated that fibroblasts cultured in native type I collagen matrix interacted with the collagen to contract it. Lapière *et al.* (Nusgens, 1984) demonstrated that fibroblasts induced more complete collagen synthesis when they were cultured in equivalent dermis than in monolayers. Bell *et al.* (1981) further demonstrated that the equivalent dermis was a good substrate for epidermalization. Fusenig *et al.* (Fusenig, 1983; Smola, 1993) set up a simplified model of reconstituted skin based on the Bell method. They cultured primary isolated keratinocytes on a reconstructed dermis and the resulting gel was lifted onto a metal grid, in order to create air-medium culture conditions. This technique allowed the formation of a well differentiated epithelium within a short time. Reconstituted skin provides an interesting model for the study of normal or pathological wound healing processes and may allow the *in vitro* reconstruction of various cutaneous pathology.

The fibroblast is the major cellular component of the normal dermis and of fibrotic tissues. During the fibrotic process, the fibroblast may develop a specific type of differentiation, and is then called myofibroblast (Gabbiani *et al.*, 1971, 1994). Myofibroblasts may be found under normal conditions, as they appear in inflammatory situations like wound healing and tissue remodeling. However, they mostly occur in a variety of pathological conditions (Skalli *et al.*, 1989), as they appear in forms of fibromatosis like Dupuytren's contracture, in breast desmoplasia carcinoma and in malignant fibroblastic tumours (Sappino *et al.*, 1990).

Both *in vivo* and *in vitro* experiments showed that fibroblasts express a series of muscle differentiation features in physiological conditions as well as under the pathological fibrotic conditions (Bouissou and Pieraggi, 1986; Pieraggi *et al.*, 1985). In this connection, the analysis of cytoskeletal proteins, especially the study of different isoforms of actin, has been useful for defining different fibroblastic phenotypes.

Actin is one of the best conserved eukaryotic proteins, and is expressed in mammals and birds as six isoforms. Four of them (α and γ-smooth muscle actin, and differentiation markers of muscle tissues, and the other two (β and γ-cytoplasmic actin) are found in all cells. Actin isoforms exhibit more than 90% overall sequence homology, but only 50–60% homology in their NH_2-terminal residues. The NH_2-terminal region of actin appears to be a major antigenic region and may be involved in the interaction of actin with other proteins such as myosin. Little is currently known about the specific functions of actin isoforms. The presence of α-SM actin in fibroblasts was reported during wound healing, suggesting that fibroblasts are equipped with a muscular element to induce the wound healing contraction (Gabbiani, 1971). Alpha-SM actin was then considered to be a marker of myofibroblast differentiation.

The presence of myofibroblasts has been reported in fibrotic skin tissue formed after ionizing radiation (Brouty-Boyer, 1991; Lefaix, 1993). This tissue is characterized by abnormal deposition of extracellular matrix proteins and fibroblasts overproliferation. An experimental model of irradiation fibrosis of the skin has been developed in pigs by Daburon *et al.* (1986). In this model, matrix deposition was due to both increased collagen synthesis (El Nabout, 1989; Remy, 1991) and decreased matrix degradation in myofibroblasts (Lafuma, 1994). Further, the myofibroblasts exhibited an abnormal cytoskeleton, with an increased number of β-actin fibers and an abnormal growth potential (Martin, 1989; Martin, 1993).

There is to date no relevant *in vitro* model of skin fibrosis. We thus decided to develop *in vitro* reconstituted fibrotic and normal skin with pig cells. We compared the differentiation patterns of normal and fibrotic fibroblasts in three types of culture: cell monolayers, equivalent dermis and reconstituted skin. In particular the expression of the cytosqueletal proteins α-SM and β-actin was assessed as a function of the type of culture. The results showed that cellular differentiation was deteriorated in the monolayers, but proceeded normally in the reconstituted dermis and skin cultures, which then constituted two relevant models of skin fibrosis.

Materials and methods

Experimental model

The study was performed on the pig experimental model set up in the LREG as previously described (Daburon, 1986; Lefaix, 1992; Lefaix, 1993). Large-White pigs were irradiated on the external side of the right thigh with a ^{192}iridium source, and were killed 6–12 months later when external healing was completed. For the present study, irradiated skin samples and the underlying fibrotic tissue were surgically removed. The control skin came from the normal flank of the same animal.

Cell cultures

Primary cell cultures were established from both normal and fibrotic skin. The biopsies were harvested and maintained in 5% Betadine PBS solution to avoid contamination. The dermis was then separated from the epidermis with a scalpel. The epidermis was incubated at 4 °C overnight in a solution composed of 0.5% trypsin (Gibco), PBS, 500 U.ml^{-1} penicillin, 250 μg.ml^{-1} streptomycin (Seromed). The dermis was lacerated at 37 °C in a solution composed of 0.25% collagenase (Boehringer), 0.5% trypsin, 500 U.ml^{-1} penicillin, 250 μg.ml^{-1} streptomycin. When all the dermis had been digested, trypsin was neutralized with foetal calf serum. The fibroblasts thus obtained were counted and frozen in 10% DMSO solution. Next day, the epidermis was scraped free of the *stratum corneum* and the basal proliferative keratinocytes were harvested. The epidermal cells were then counted and frozen

in 10% DMSO solution. Keratinocytes were plated at a density of 10–20 10^3 cells.cm^{-2} on dishes containing feeder-layers consisting of irradiated normal human skin fibroblasts (Green, 1988). A specific medium was used for the culture of primary keratinocytes, the HD medium: a mixture of 2/3 Ham's F12 and 1/3 DMEM containing 1g.l^{-1} glucose supplemented with 5% FCS, 100 U.ml^{-1} penicillin, 50μg.ml^{-1} streptomycin, 5 μg.ml^{-1} insulin (Sigma), 200 μM adenine (Sigma), 10^{-7} M cholera glutamine (Gibco). For all experiments, primary keratinocytes and fibroblasts (passage 1) were used. Fibroblasts were cultured in DMEM medium containing 4.5 g.l^{-1} glucose supplemented with 10% FCS, 100 U.ml^{-1} penicillin, 50 μg.ml^{-1} streptomycin, 1 M hepes and 200 mM glutamine.

Equivalent dermis model culture

Type I collagen was extracted from rat tail, lyophilized and stored at 4 °C. For cell culture, the collagen was suspended in a 0.1% acetic acid solution, to obtain a 6 mg.ml^{-1} concentration. Eight volumes of this icecold suspension were mixed with one volume of 10x Hank's balanced saline solution and neutralized with NaOH under gentle stirring. One volume of an FCS solution containing 100.10^3 fibroblasts was added. One milliliter of the fibroblast-collagen matrix was poured into each well of 24 well plates. The gels thus formed were stored for 1 hour in a humid incubator for polymerization, and were then immersed in FAD medium composed of 1/3 Ham's F12 and 2/3 DMEM containing lg.l^{-1} glucose supplemented with 10% FCS, 100 U.ml^{-1} penicillin, 50 μg.ml^{-1} streptomycin, 10^{-7} M cholera toxin (Sigma), 0.4 μg.ml^{-1} hydrocortisone (Sigma), 50 mg.ml^{-1} ascorbic acid (Sigma), 1 M hepes (Gibco) and 200 mM glutamine (Gibco). Next day, the gels were transferred onto a metal grid to create an air-medium interface. The collagen-gels were cultured for two weeks in FAD medium changed every two days. Under these conditions, the cells were nourished by diffusion of the nutriments through the gel.

Reconstituted skin culture model

The same protocol was used to insert fibroblasts into the collagen gels. Then, when the cell-populated gels were in the 24 well plates, 500.10^3 keratinocytes were immersed in FAD culture medium, seeded on the gels and incubated for 24 hours. The gels were then lifted to the air-medium interface and cultured for two weeks.

Hemalun eosin staining and indirect immunofluorescence

After two weeks in culture, the reconstituted skins were mounted in tissue-Tek (Miles Inc, USA) and snap-frozen in liquid-nitrogen. Sixteen μm sections were cut in a Minotome cryostat at –20°C. Frozen sections were fixed with paraformaldehyde (0.5%, 30 min) and stained with hemalun-eosin. For immunohistochemistry, sections were also permeabilized with triton (0.1%, 10 min), washed in PBS and saturated with a PBS-BSA (2%) solution. Sections were then incubated with the primary antibody overnight (0.2% BSA solution) at 4 °C. They were washed 3 times in PBS-BSA (0.2%) and incubated for 45 min with the secondary antibody (0.2% BSA solution). Sections were then mounted in PBS-glycerol (50%) and examined with an epifluorescence microscope (Olympus BH2).

We tested each antibody to obtain the concentration that gave maximal specific fluorescence and minimal background fluorescence. Primary antibodies to epidermis differentiation markers: involucrin (Sigma mouse monoclonal anti-human n^o I-9018, dilution 1/300); cytokeratin (Sigma mouse monoclonal anti-bovine n^o C-7284, dilution 1/300). Primary antibodies to dermis markers: α-smooth muscle actin (Sigma mouse monoclonal n^o A-2547, dilution 1/500); β-actin (Sigma mouse monoclonal n^o A5316, dilution 1/500). Secondary anti mouse FITC antibody (Immunotech, dilution 1/200).

Western-blot analysis

For the three types of culture, monolayers, equivalent dermis and reconstituted skin, the fibroblast layer was treated with lysis solution (Tris 90 mM, SDS 4%, β mercaptoethanol 2%, water), to extract the total proteins. The protein concentration of the samples was determined according to Bradford (Bradford, 1976). Proteins (5–10 μg) were separated by electrophoretic migration in 12% acrylamide gel. They were then transferred onto nitrocellulose membranes by wet electrotransfer. A second control gel was stained with Coomassie blue to assess the equality of protein loading. Furthermore, we tested the quality of the transfert by Ponceau red staining, that also gave us the confirmation of loading equality. The membranes were then saturated with a PBS-Tween-Milk (5%) solution and incubated overnight at room temperature with the primary antibody (α-smooth muscle actin 1/1000; β-actin 1/1000) diluted in the saturation solution. The mem-

branes were incubated for 45 min with the secondary peroxidase labeled antibody (1/1000). The membranes were washed in PBS-Tween and the protein expression was revealed with the ECL kit (Amersham) on autoradiographic films.

Semi-quantitative densitometric analysis was performed on Molecular Analyst (Biorad). We compared the levels of protein within the same culture conditions. Because in the case of monolayer culture the proteins are only cellular products whereas in the case of the 3-D models we have a mix of cellular protein and collagenous matrix proteins.

Cell proliferation

Equivalent dermis composed of fibrosis myofibroblasts and normal fibroblasts were cultured as previously described. A kinetic of counting was performed at days 3, 10 and 14 after the beginning of the 3-D culture model. We could not perform this study on reconstituted skin because early in the culture we could not separate fibroblasts from keratinocytes as the epidermis is not well differentiated. Eight samples were studied each time. Each sample was washed two-times in PBS to remove the medium, and immersed in 1 ml of 2 mg.ml^{-1} Collagenase (Boehringer)-PBS solution to digest the collagen matrix. Samples were incubated for 20 min at 37 °C until complete dissolution occurred. After 10 min centrifugation at 1000 rpm, each sample was resuspended in 100 μl of PBS and counted in Malassez and Neubauer chamber blindly by two different persons.

Statistical analysis

Data are expressed as means ± SE. Analysis of variance and Student's t test were used to assess the significance of differences; $p < 0.05$ was considered significant.

Results

Culture in monolayers

The expression of α-SM actin in normal fibroblasts was first studied by immunofluorescence. Surprisingly, we found that, when cultured in monolayers, normal fibroblasts expressed α-actin (Figure 1a). Western-blot analysis (Figure 3a) showed a single α-actin band at 42 kD in normal fibroblasts, thus confirming the immunocytochemistry staining. When the same experiments were conducted on fibrosis myofibroblasts, marked expression of α-actin was observed (Figure 1b). Fluorescent microscopy showed a dense microfilamentous actin system throughout the cell, very strongly labelled by the anti α-SM actin antibody. Myofibroblast expression of α-actin was confirmed by western-blot analysis. Figure 3d shows the single 42 kD actin band in myofibroblasts. The difference was quantified. The mean ratio of α-actin for myofibroblasts *versus* normal fibroblasts was 1.55 (SE = 0.13, n = 9), thus showing no significant difference between the two cell types.

Immunofluorescence and western-blot allowed detection of β-actin in normal fibroblasts, as shown in Figures 1c and 4a. The expression of β-actin was also detected in myofibroblasts by both techniques (Figures 1d and 4d). The mean ratio of β-actin for myofibroblasts *versus* normal fibroblasts was 0.26 (SE = 0.05, n = 5). However, this decreased expression in myofibroblasts was not observed by immunocytochemistry. It was not possible to study the keratinocytes cultured in monolayers, because their growth on an irradiated fibroblast feeder layer created many artefacts.

Equivalent dermis

Expression of α-SM actin and β-actin

The equivalent dermis was only studied by western-blotting. For the normal fibroblasts cultured in equivalent dermis, no expression of α-SM actin protein was detectable by western-blot analysis (Figure 3b). The myofibroblasts cultured in the equivalent dermis model kept their differentiation features, since they expressed α-actin as shown in Figure 3e.

Beta-actin was expressed in normal fibroblasts, but at a very low level, since one hour of overexposure was necessary to detect the 42 kD (Figure 4b) where 1–2 min were usually sufficient. Figure 4e shows that myofibroblasts overexpressed β-actin. The β-actin band appeared after a brief exposure of 1.5 min, whereas one hour of overexposure necessary for the normal fibroblasts, resulted in a saturated signal (Figure 4e). The mean ratio of β-actin for myofibroblasts *versus* normal fibroblasts showed a significant difference between the two cell types: 8.24 (SE = 0.95, n = 5).

Analysis of cell number

We studied cell proliferation in the time course of the 3-D culture, in order to assess whether the cellular density

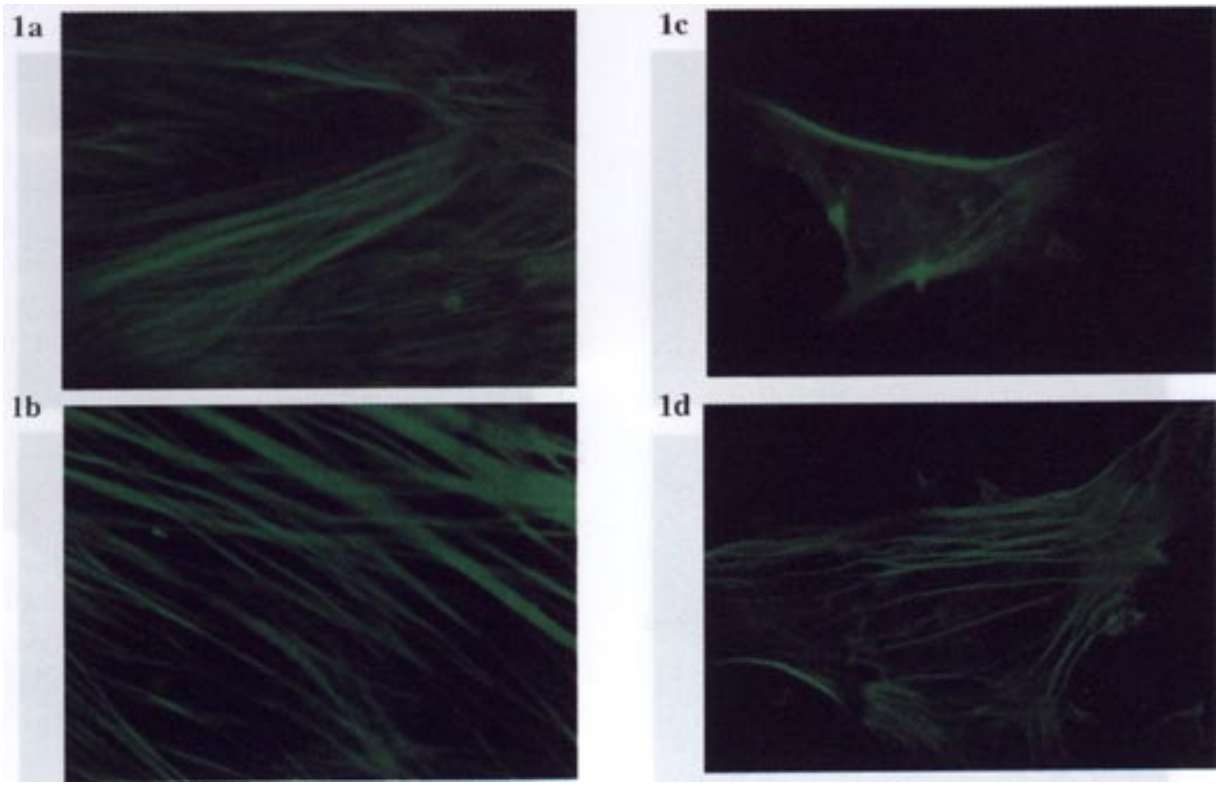

Figure 1. Immunohistochemical study of fibroblasts differentiation in monolayer (Obj. X40). 1a,1c: Normal fibroblasts cultured in monolayers. 1a: Positive α SM actin staining. 1c: Positive β actin staining. 1b, 1d: Fibrosis myofibroblasts cultured in monolayers. 1b: Positive α SM actin staining. 1d: Positive β actin staining.

Table 1. Number of cells in equivalent dermis according to the time in culture

Cell type	Day 0 n = 8	Day 3 n = 8	Day 10 n = 8	Day 14 n = 8
Normal fibroblasts	100.10^3	53.10^3 $\pm$ 6	134.10^3 $\pm$ 7	164.10^3 $\pm$ 12
Fibrosis myofibroblasts p >	100.10^3	75.10^3 $\pm$ 6 0.001	113.10^3 $\pm$ 6 0.001	128.10^3 $\pm$ 16 0.001

The equivalent dermis was cultured as described in Materials and Methods. 100.10^3 fibroblasts were seeded in the collagen matrix at day 0 for each cell type. Cells were counted at day 3, 10, 14 after collagenase digestion of the gels. Statistical differences between normal fibroblasts and fibrosis myofibroblasts were performed as described in Materials and Methods (n = number of samples).

and proliferation could be involved in the difference observed in actin expression.

Table 1 shows a decrease of the total cell number within the first days of the 3-D culture (Day 3). Then proliferation occurred between day 4 and 10. During the last four days in culture, proliferation reached a plateau, so that the fibroblasts entered a quiescent phase. As immunofluorescence and western-blot were performed at this stage, proliferation was probably not a major factor involved in the normalization of cellular phenotypes between monolayers and 3-D models.

The growth curve was not exactly the same in the two cell types. Myofibroblasts were less affected by the culture conditions at 3 days, but exhibited a slower proliferation between day 4 and 10 than the normal fibroblasts.

Reconstituted skin

Epidermis

The first step was to characterize this model for normal pig skin cells and set up standard culture conditions in order to obtain reproducible reconstituted tissue. This study was done on frozen sections. Hemalun eosine staining (Figures 2a and d) showed that both nor-

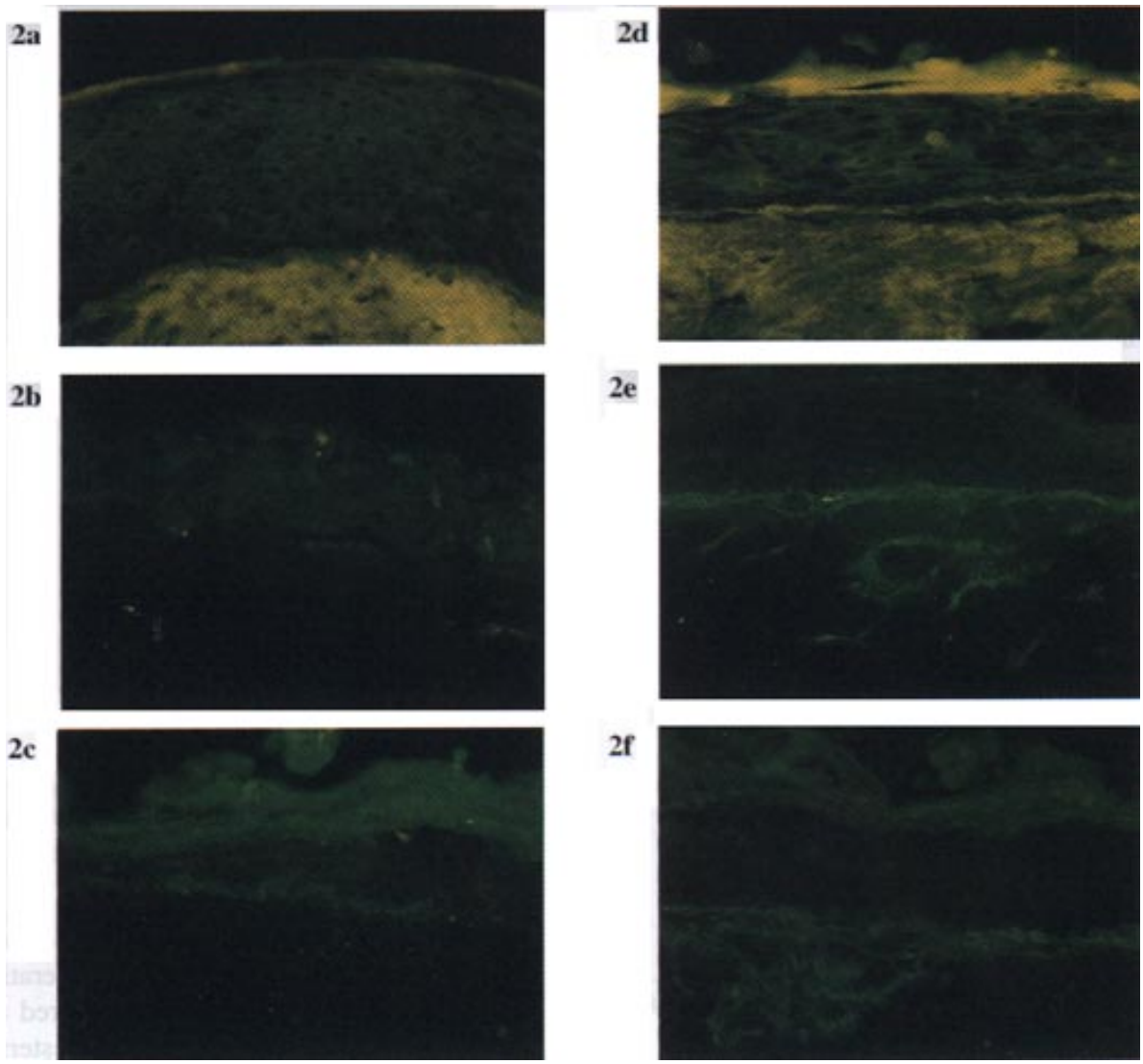

Figure 2. Immunohistochemical studies of normal and fibrosis reconstituted skin (Obj. X20). 2a, 2b, 2c: Reconstituted skin composed of normal fibroblasts cultured in a type I collagen matrix overlaid by normal keratinocytes. 2d, 2e, 2f: Reconstituted skin composed of fibrosis myofibroblasts cultured in a type I collagen matrix overlaid by scar keratinocytes. 2a: Hemalun-eosin staining, observed in UV light, of normal reconstituted skin. The keratinocytes autofluorescence allowed the visualization of a well differentiated epidermis. 2b: Negative α SM actin staining in dermis and epidermis of normal reconstituted skin. 2c: Positive β actin staining at low level in the dermis of normal reconstituted skin. 2d: Hemalun-eosin staining, observed in UV light, of fibrosis reconstituted skin. 2e: Positive α SM actin staining in the dermis of fibrosis reconstituted skin. 2f: Positive β actin staining in the dermis of fibrosis reconstituted skin.

mal and scar keratinocytes differentiate into epidermis composed of a *stratum basal, stratum spinosum, stratum granulosum* and *stratum corneum*. Although the normal keratinocytes gave a thicker and better organized epidermis than did scar keratinocytes, 4 to 7 layers were observed for the "scar" epidermis. We observed that the best conditions for skin differentiation depended on the concentration of type I collagen (6 mg.ml^{-1}) and on the number of keratinocytes (5.10^5) lying on the collagen-fibroblast matrix. We then checked the good differentiation of the epidermis by studying cytokeratin and involucrin production in both normal and scar epithelium, as previously described in the literature. We detected expression of cytokeratins in the upper *stratum* of the epidermis. We also detected involucrin, which expression is restricted to mature

epithelium (data not shown). We did not observe any differences between normal and scar keratinocytes as regards their expression of cytokeratins and involucrin after two weeks of culture.

Normal skin fibroblasts
We then studied the expression of the selected differentiation markers of the dermis. Immunofluorescence (Figure 2b) and western-blotting (Figure 3c) revealed that the normal fibroblasts did not express α-SM actin, which is known to be a muscle cell differentiation marker, but expressed β-actin (Figures 2c, 4c).

Fibrotic skin myofibroblasts
We studied the expression of α-SM actin in the reconstituted skin consisting of pathologic fibroblasts. Immunofluorescence and western-blotting (Figures 2e and 3f) showed that myofibroblasts retained their muscle differentiation features, and expressed a high level of α-actin.

We studied the expression of β-actin, whose mRNA was previously reported to increase in pig fibrotic tissue. Here, immunofluorescence and western-blotting of reconstituted skin confirmed this result at the protein level (Figures 2f and 4f), as myofibroblasts overexpressed β-actin. The mean ratio of β-actin for myofibroblasts *versus* normal fibroblasts was 2.68 (SE = 0.75, n = 5), thus confirming immunofluorescent evaluation. The differences observed between equivalent dermis and reconstituted skin for densitometric analysis suggested that the keratinocytes were involved in the regulation of fibroblasts phenotypes.

Discussion

To study the mechanisms of fibrotic development, we attempted to set up culture models of skin fibrosis. Thus the differences between normal and fibrotic fibroblasts were characterized in various culture models concerning two myofibroblastic differentiation markers: α-smooth muscle actin and β-actin. Fibroblasts have been extensively studied *in vivo* and *in vitro*. Morphological differences between *in vivo* and cultured human fibroblasts suggest that monolayer cell culture induce alterations of the fibroblast phenotype. Thus according to Sappino *et al*. (1990), human fibroblasts *in vivo* display few microfilaments and intermediate filaments and do not establish contacts among themselves. By contrast, fibroblasts cultured in monolayers exhibit a flattened and polarized shape, possess numerous stress fibers and are interconnected by gap junctions. According to the literature (Brouty-Boyer, 1991; Gabbiani, 1994; Skalli, 1990), normal human fibroblasts do not express α-SM actin.

Surprisingly, we found here that normal pig fibroblasts did express α-SM actin when cultured in monolayers in exponentially growth phase and even at confluency. Normal fibroblasts in the skin are quiescent, but when cultured in monolayers, they became proliferative and behave more like the activated fibroblasts found in the normal wound healing process than like quiescent cells of the dermis. At confluency, the pig fibroblasts were still activated and synthesized numerous stress fibers. Although few studies have been performed on pig fibroblasts, our results suggest that monolayer culture triggered the expression of α-SM actin in normal pig fibroblasts. Furthermore, Ehrlich *et al*. (1994) demonstrated that normal human fibroblasts and fibroblasts isolated from keloids expressed α-SM actin when cultured *in vitro*, but not *in vivo*. *In vitro* these cells were stimulated to produce α-SM actin by serum components.

In the present investigation, the pig fibroblasts isolated from fibrotic tissue exhibited the morphology of myofibroblasts (Martin, 1989; Lefaix, 1993). As expected, these myofibroblasts expressed α-SM actin, even in monolayer cultures. With regard to β-actin expression, when fibrotic and normal fibroblasts were cultured in monolayers, β-actin expression was slightly reduced in the myofibroblasts. The adaptive expression of α-SM actin in normal fibroblasts and the weak expression of β-actin in myofibroblasts cultured in monolayers raise a serious problem for the interpretation of future experimental results. In addition, the study of fibroblast responses to anti-fibrotic agents cannot be developed in cells which display such abnormal differentiation.

To overcome this problem, we decided to develop two 3-D culture models: equivalent dermis and reconstituted skin. We found that the simple model of the equivalent dermis allowed the normalization of both the expression of α-SM actin and β-actin. The same result was obtained with the reconstituted skin model, which is more complex, but has significant advantages. It allowed the study of epithelial and mesenchymal cells interactions. This model also allowed an histological localisation of cellular products, that could be of great interest in the study of diffusible factors, like cytokines. In the reconstituted skin model, we cultured normal fibroblasts and keratinocytes, in order to

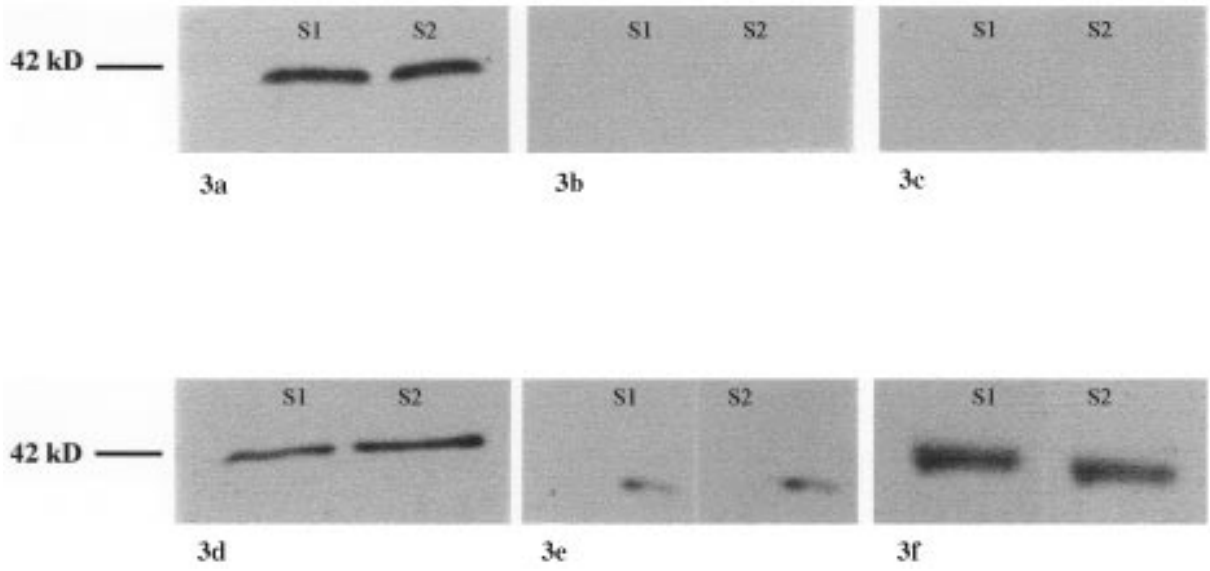

Figure 3. Western blot analysis of α-SM actin expression in the three culture models. The S1 and S2 bands correspond to the loading of two separate samples obtained under the same experimental conditions. 3a, 3b, 3c: Normal fibroblasts cultured in monolayer a, equivalent dermis b and reconstituted skin c. 3d, 3e, 3f: Fibrosis myofibroblasts cultured in monolayer d, equivalent dermis e and reconstituted skin f. 3a: Detection of the α-SM actin band at 42 kD in normal fibroblasts. 3d: Detection of α-SM actin in myofibroblasts. 3b: No detection of α-SM actin in normal fibroblasts. 3e: Detection of α-SM actin in myofibroblasts. 3c: No detection α-SM actin in normal fibroblasts. 3f: Detection of α-SM actin in myofibroblasts.

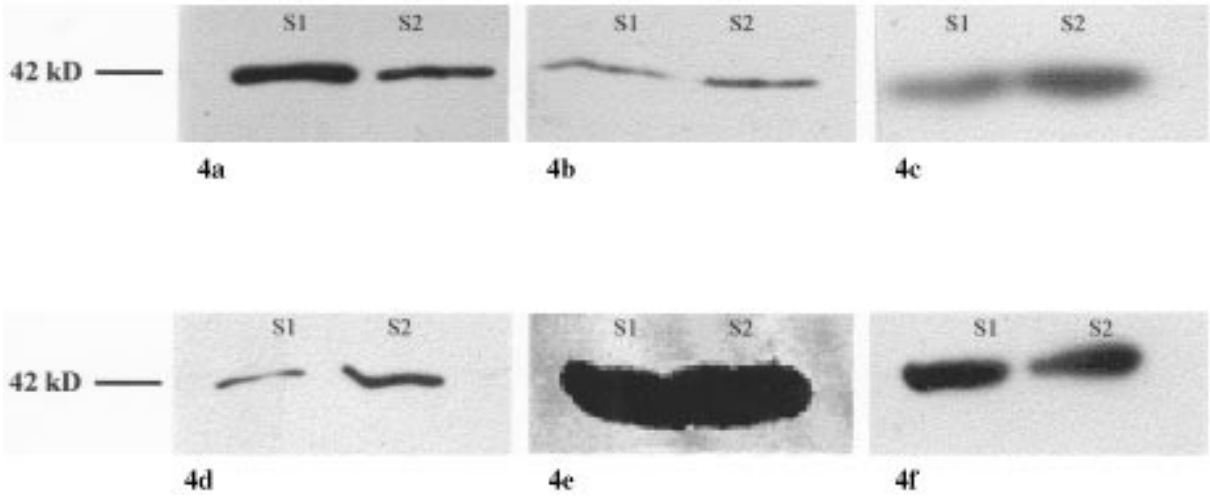

Figure 4. Western blot analysis of β-actin expression in the three culture models. The S1 and S2 bands correspond to the loading of two separate samples obtained under the same experimental conditions. 4a, 4b, 4c: Normal fibroblasts cultured in monolayer a, equivalent dermis b and reconstituted skin c. 4d, 4e, 4f: Fibrosis myofibroblasts cultured in monolayer d, equivalent dermis e and reconstituted skin f. 4a: Detection of the β-actin band at 42 kD in normal fibroblasts. 4d: Detection of β-actin in myofibroblasts. 4b: Detection of β-actin in normal fibroblasts after one hour of overexposure. 4e: Detection of the saturated band in myofibroblasts after one hour of overexposure. 4c: Detection of β-actin in normal fibroblasts. 4f: Detection of β-actin in myofibroblasts.

obtain “normal” reconstructed skin. In addition, we succeeded in partially mimicking fibrosis in this skin reconstituted *in vitro*, by culturing fibrosis myofibroblasts overlaid with keratinocytes isolated from fibrotic skin. In both cases, a well differentiated epithelium was obtained after only two weeks of culture.

How did these normalizations occur? It is assumed that the fibroblasts behave in a different manner whether they are cultured in monolayers or in collagen gels. The collagen matrix allowed a three dimensional conformation of the cells, closer from the *in vivo* conditions than the growth on plastic dishes. We investigated whether cell proliferation and density could be involved in this mechanism or if the collagen environment and the cellular differentiation patterns were the main factors involved.

We realized a study of cell proliferation during the time course of the 3-D culture. Normal fibroblasts and myofibroblasts exhibited similar growth curves. After a phase of proliferation, both cell types were in a state close to quiescence, as already shown by Bell *et al*. (1983), and exhibited various phenotypes. Concerning the monolayers, we studied fibroblasts at confluency in order to avoid modifications due to cell proliferation and to mimick again the quiescent state of the cells in the skin. However, even at confluency, normal fibroblasts cultured in monolayer expressed α-SM actin. These results suggest that, in our models, both actin expression and cellular proliferation are controlled by the collagen matrix. The cellular membrane interacted directly with this collagen matrix, that constituted a microenvironnement acting directly on cellular behaviour. Furthermore, the matrix avoided the intercellular contacts and gap junctions observed in monolayer culture.

We propose that the main factor governing cell differentiation is the genetic programmation acquired by the cells in the tissue. The 3-D culture environment allows the persistance of this programme, while the monolayer environment induces alteration of this programme. Further studies are in preparation to investigate whether the modulation of cytoskeleton proteins could be linked with cellular motility and contraction of the collagen gel.

Both our 3-D models allowed the study of radiation-induced fibrosis *in vitro*, provided good extrapolations to *in vivo* conditions and avoided certain culture artefacts. The models can be quickly constructed and are easily reproducible. Thus they constitute a very good alternative to animal experimentation. In addition, they can be rendered more complex by adding other skin cells like endothelial cells, melanocytes or inflammatory cells to the culture medium.

In fibroblasts, the mechanisms leading to the development of cytoskeletal features similar to those of smooth muscle cells, including those which regulate the *in vivo* and *in vitro* appearance of α-SM actin, are not yet known. The most likely candidates are cytokines and growth factors. TGF β1 might be one of these factors, as we published that this cytokine has a key role in the cascade of events that leads to radiation-induced skin fibrosis (Martin, 1993). Further studies of the factors regulating the myofibroblast phenotype will probably be useful for understanding myofibroblast behaviour and possibly for modifying this behaviour by anti-fibrotic treatments.

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