Proliferation characteristics and polyploidization of cultured myofibroblasts from a patient with fibroblastic rheumatism

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Fibroblast-like cells were obtained from a nodule of a patient with fibroblastic rheumatism, and grown in culture for different times (from passage 3 to 21). These cells as well as the fibroblasts taken from an unaffected skin area (controls) of the same patient, have been investigated by fluorescence microscopy, cytochemical methods and cytometry, to evaluate their cytodifferentiation features and cytokinetic characteristics. In addition, in low-passage cultures, the secretion of collagen and of non-collagenic proteins was evaluated using electrophoretic techniques. The immunolabeling with antibodies against sm-specific α-actin (which was taken as a marker of myofibroblasts) showed that, already in low-passage cultures, the percentage of myofibroblasts was higher in the nodule-derived cell populations, and progressively increased with increasing passages. This suggests that myofibroblasts have higher proliferation potential than control fibroblasts. Myofibroblasts were also found to undergo polyploidization and hypertrophy, especially in high-passage cultures. Based on these results, it may be hypothesized that in fibroblastic rheumatism the development of the typical nodules could depend on the intrinsic capability of myofibroblats of proliferating faster than normal fibroblasts and of becoming polyploid and hypertrophic. Nodule-derived cells in culture synthesized slightly less collagen and non-collagen proteins than did the control fibroblasts; this suggests that the increased fibrosis observed in nodules *in situ* could be likely dependent on a reduced degradation of the extracellular matrix components.

Key words: fibroblastic rheumatism, cell cultures, myofibroblasts, cell proliferation, cytochemistry, collagen synthesis.

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Fibroblastic rheumatism (FR) is a very rare disease, which mostly affects young people (Lacour et al., 1993). Since its firts description (Chaout et al., 1980), 19 cases only have been reported: 11 in France (Chaout et al., 1980; Vignon-Pennamen et al., 1986; Crouzet et al., 1982; Levigne et al., 1990; Barbaud et al., 1990; Leclech et al., 1990; Lacour et al., 1993; Vittecoq et al., 1996; Masson et al., 1997), 2 in Italy (Schiavon et al., 1982; Taccari et al., 1987) and the United States (Kanzler et al., 1995), one in Great Britain (Ostlere et al., 1994), Australia (Romas et al., 1997), Canada (Fam et al., 1998) and Morocco (Chkirate and Job-Deslandre, 2001) respectively.

FR is characterized by cutaneous nodules and arthritis followed by a cronic phase which entails sclerodactyly and dermic fibrosis mimicking scleroderma (Fam et al., 1998).

Ultrastructural analyses showed that nodule fibroblasts in FR have the structural characteristics of myofibroblasts (Taccari et al, 1987; Hernandez et al, 1989; Barbaud et al., 1990; Lacour et al, 1993), and the increase in their number is likely due to a higher proliferation activity, although no clearcut proof exists in the literature at this subject (Lacour et al., 1993). The hyperplastic activity of fibroblasts would also affect the secretion of collagen(s) and of non-collagenic proteins finally leading to alterations in the extracellular matrix (Lacour et al., 1992, 1993). At present it is still debated whether nodular fibroblastic hyperplasia in patients with FR is merely related to a local stimulatory milieu as suggested by Fam et al.(1998) or it also reflects persistent changes in the phenotype of the fibroblasts involved.

The aim of the present investigation was to assess whether the fibroblasts from nodules of a patient with FR actually exhibit higher proliferation capability, how long this characteristics is eventually preserved during long passages in culture, and whether it actually correlates with the cytodifferentiation

features (the immunopositivity for smooth-muscle specific α-actin was taken as the marker for myofibroblats). In the attempt to elucidate these points, fibroblast-like cells were obtained from a nodule and the unaffected skin of a 23 years old male patient, and were grown in culture for different passages to be analysed for their morphology, cytodifferentiation characteristics, and proliferation capability: to do this, light microscopy, cytochemical methods and cytometry were used. In addition, secretion of collagen and of non-collagenic proteins was evaluated by electrophoretic techniques.

Materials and Methods

After informed consent, tissue fragments of about 10 mm^3 were taken from the nodule and unaffected skin of a 23 years old male patient undergoing skin biopsy as routine diagnostic procedure for FR. For sake of simplicity, the fibroblasts from the unaffected skin will be defined as controls.

To obtain primary cultures of fibroblast-like cells, the tissue fragments were minced with sterile scissors and placed in 25 cm^2 flasks with 1 mL of complete Dulbecco's modified Eagle's medium (D-MEM) containing 20% heat-inactivated fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin; the medium was replaced daily for about two weeks, then the tissue fragments were removed (and used to obtain new primary culture) while the adhering cells were detached by mild trypsinization (0.25% trypsin in PBS containing 0.05% EDTA) and planted either on flasks or on glass slides in Petri dishes (this was considered as the passage 1). Cells were grown in complete D-MEM medium containing 10% FBS at 37°C in a humidified 95% air, 5% CO$_2$ atmosphere and allowed to grow at confluence, when they were detached as above and divided into three flasks. Cells were studied from passage 3 to 21.

Cytochemical and cytometric analyses

For this part of the investigation, which was mostly performed by light microscopy, cells at the different passages were planted on glass slides and used after 48 hr.

Detection of the myofibroblatic phenotype. Cells were fixed for 20 min with 70% ethanol at -20°C, washed with PBS and permeabilized for 10 min with PBS containing 0.1% albumin and 0.05% Tween-20. Cells were then incubated with an FITC-conjugated monoclonal antibody recognizing smooth-muscle (sm) specific α-actin (Sigma Chemical Co., St. Louis MO, USA: dilution 1:250 for 1 hr at room temperature, RT). Some cell samples were also labeled for fibrillary actin with FITC-conjugated phalloidin (Molecular Probes, Space, Milan, Italy) for 1 hr as suggested by the manufacturer. After the fluorescent labeling procedures, cells were finally counterstained for DNA with propidium iodide (PI, 1 μg/mL in PBS) for 5 min, and mounted up-side-down on glass slides, in a drop of Mowiol (Calbiochem, Inalco S.p.A., Milan, Italy).

Identification of S-phase cells by bromodeoxyuridine (BrdU) incorporation. To estimate the percentage of S-phase cells, the cultures were labeled with 4×10^{-5} M BrdU for 30 min; cells were then fixed in 70% cold ethanol as above, washed with PBS and treated with 3N HCl at RT for 30 min to denature DNA partially. BrdU incorporation was revealed with an anti-BrdU antibody (Exbio Praha a.s., Prague, CR, dilution 1:20) followed by an FITC-conjugated anti-mouse-IgG antibody (DAKO, Glostrup, Denmark, dilution 1:20). Both antibodies were diluted in PBS containing 0.1% bovine serum albumin and 0.5% Tween 20 (Sigma Chem. Co.). The experiments were performed in duplicate on control and nodule-derived cells. For fluorescence microscopy we used an Olympus BX50 photomicroscope under the following conditions: 450-480 nm excitation filter (excf) 500 nm dichroic mirror (dm) and 515 nm barrier filter (bf) for FITC, or 540 nm excf, 580 nm dm, and 620 nm bf for Evan's blue and PI. Images were recorded with an Olympus Camedia C-2000 Z digital camera and stored on a PC by the Olympus software, for processing and printing. The percentage of BrdU-positive cells was estimated on ten randomly-selected fields for each slide.

Flow cytometric measurement of DNA content. Cell cultures at some passages were trypsinized, and immediately stained for 30 min with 50 μg/mL PI in distilled water containing 100 U/mL of RNase type A and 0.0015% Nonidet-P40 (all reagents from Sigma Chemical Co.). Flow cytometric measurements were taken with a FACStar (Becton Dickinson, San José, CA, USA) equipped with a laser excitation (power 200 mW) at 488 nm and a 610 nm long-pass filter for the red fluorescence signals. The percentages of BrdU-positive (S-phase) cells and those of BrdU-negative cells in the different cycle phases were evaluated by rectangu-

lar-region analysis on dual-parameter scatter-grams. At least 20,000 cells were contained in the gated regions used for calculations.

Karypotype analysis

Cells were arrested in metaphase by Colcemid treatment (0.5 µg/mL for 4 hr). The whole cell populations were harvested by mild trypsinization (as reported above), centrifuged, resuspended in 0.56% KCl for 15 min, centrifuged and fixed with a 3:1 mixture of methanol:acetic acid for 30 min at 4°C. Cells were finally spread onto glass slides, air-dried and stained with 0.1 µg/mL Hoechst 33258 in 0.1M phosphate buffer pH 6.8 for 15 min. Slides were observed and scored for polyploid metaphases in fluorescence microscopy (330-385 nm excf, 400 nm dm, and 420 nm bf).

Biochemical analysis

Nodule and control cells at the 4th passage were plated at a density of 30,000 cells per well in 24-well culture plates (COSTAR) and maintained in 10% FBS for 24 h. The medium was then replaced by pre-labelling medium:DMEM, supplemented with ascorbic acid (100mg/mL) for 4 h. The medium was then replaced with pulsing medium:DMEM supplemented with ascorbate, containing 40 mCi/mL 2,3 3H proline and cultures incubated for 20 h. After labelling the covered plate was removed and heated to 90°C in a water bath for 10 min. The plate was then frozen and thawed three times to disintegrate the cells; the contents of each well mixed by refluxing, to ensure homogeneity, and aliquot (0.2 mL) removed for DNA analysis and stored at -25°C until needed. The remaining contents of each well were transferred to eppendorf tubes. Each well was rinsed with 1 mM proline (100 mL) and the wash added to the extract. Type I collagen (20 mL of 1 mg/mL) as carrier was added to each sample and the contents of each vortexed. A solution of 50% cold trichloroacetic acid (TCA) was then added and the tubes kept on ice for 10 min, centrifuged and supernatants (containing free isotope) were carefully removed and discarded. The pellet was resuspended in 0.9 mL cold 1 mM proline overnight at 4°C, 0.1 mL cold 50% TCA was added and placed on ice for 10 min. The washing/centrifugation step was repeated twice more to ensure complete removal of unincorporated 3H proline. The pellet was washed with 1 mL cold ethanol:ethyl ether (3:1) to extract the TCA. After centrifugation the supernatant was removed and the ethanol:ethyl ether extraction repeated. The pellets were air dried at room temperature and then suspended in Hepes buffer (pH 7.2), 1.25 mmol NEM 0.25 mmol $CaCl_2$ in a final volume of 0.5 mL and samples incubated at 37°C for 90 min. Samples were then chilled to 4°C and 0.5 mL 10% TCA added. After centrifugation the supernatant was transferred to a scintillation vial and each sample was rinsed again with 0.5 mL 5% TCA. The second wash was added to the first (incubation blank). The TCA was removed from the wells by two ethanol:ether washes and after evaporation the incubation cocktail containing 10 units purified bacterial collagenase (Worthington) in 0.01 mL of 0.05M Tris (pH 7.6) and 0.005M $CaCl_2$ was added to each eppendorf tube and incubated for 90 min at 37°C. The undigested protein was precipitated with TCA twice and the supernatants were placed in scintillation vials for radioactivity determination (radioactive collagen fraction). The remaining non-collagen protein in the pellets was dissolved in 0.5 mL of 0.2M NaOH and transferred to vials. Each well was rinsed with 1 mL of 0.2M HCl and added to the first supernatant to be counted (non collagen fraction). DNA determinations were performed according to Labarca and Paigen (1980).

Results

At light microscopy, in low (3 to 5) passage cultures, the nodule cells showed typical fibroblast morphology, and were fully similar in their shape and size to the controls (not shown). However, the growth curves demonstrate that fibroblasts from the nodule grew about four times faster than those from unaffected skin (Figure 1). At day 10, the number of fibroblasts from the nodule was 16-fold greater with respect to the number of fibroblasts plated. In the case of fibroblasts from the unaffected skin, the increase was nearly 4-fold. This was confirmed by the experiments of BrdU incorporation in cell cultures from passage 3 to 21 (Figure 2): as expected, by increasing passages the percentage of S-phase cells decreased progressively (due to cell aging in culture), but was always significantly larger for nodule-derived cells.

All the cells in both normal and nodule-derived cultures were positive for fibrillary actin (Figure 3, upper row). On the contrary, the immunolabeling with antibodies against sm-specific α-actin (which
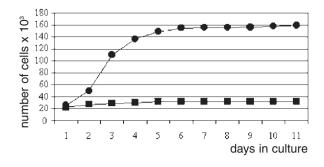
Figure 1. Growth curves of fibroblasts from the nodule (●) or the unaffected skin (■) of the FR patient.

Figure 2. Percentage of BrdU-positive (S-phase) cells in cultures of fibroblasts from the nodule (●) or the unaffected skin (■) of the FR patient.

is also expressed by myofibroblasts, but not by fibroblasts) showed that, already in low-passage cultures, the percentage of myofibroblasts was higher in the cell populations from the nodule than from the unaffected skin (Figure 3, lower row). Interestingly enough, the percentage of immunopositive cells increased in nodule cultures with increasing passages, thus suggesting that myofibroblasts have higher proliferation potential than fibroblasts.

The cytometric measurements of DNA-content showed that almost all fibroblasts from unaffected skin are diploid in low- as well as in high-passage cultures (Figure 4a,b), whereas the fraction of cells with DNA amounts exceeding the 4C values apparently increased from passage 10 onwards in nodule-derived cultures (Figure 4a',b'), thus suggesting that polyploidization occcured: karyotype analyses (not shown) confirmed that, already at passage 6-7, tetraploid mitoses were about 10% (9.6 ± 1.7) in nodule-derived cultures whereas they were less that 4% in controls. Consistent with this increase in tetraploids, cells from the nodule also became larger than controls, as shown by the dual parameter scattergrams of DNA content versus the low-angle light-scatter values (Figure 4c,c').

The electrophoretic analysis showed that there was no difference in collagen α-chain migration in the case of collagens synthesized by fibroblasts from the nodule or unaffected skin (data not shown) and the ratio between collagens types I and III was normal (Table 1).

The fibroblasts deriving from the nodule site of the FR patient synthesize slightly less collagen and non collagen proteins than do fibroblasts taken from the unaffected skin area. This appears to be in agreement with previously reported findings (see Lacour et al., 1993).
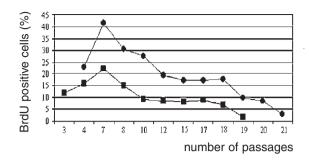
Table 1. The percentage of type III collagen was determined by densitometric scanning of the bands of α-collagen chains.

SAMPLE	RATIO TYPE III/I+III MEDIUM	RATIO α $^1/_2$ MEDIUM
nodule-derived cells	0.125	1.90
cells from unaffected skin	0.100	1.84

Normal values: ratio type III/types I + III collagen = 0.09 – 0.15.
Normal values: ratio $\alpha^1/_2$ chains of type I collagen = 2.

Discussion

Fibroblastic rheumatism is a scleroderma-like disorder involving skin and joints. However there are several differences between FR and scleroderma as far as clinical features and immunological profile (Fam et al.,1998). The present study shows that the behaviour in culture of skin fibroblasts in FR nodules is also quite different from that of skin fibroblasts in scleroderma. Cultured fibroblasts derived from the skin of patients with scleroderma are characterized by both increased proliferative activity and increased collagen production (Varga and Jimenez 1996). The results of our investigation demonstrate that in cell cultures from the nodule of a patient with FR the proliferative activity is highly increased while the collagen production is slightly decreased as compared with fibroblasts from unaffected skin. Thus the fibrotic changes occurring *in vivo* in FR are likely independent of an increased protein synthesis rate in affected fibroblasts.

The hyperproliferative phenotype found in FR cells shows some peculiar features; first of all, it is related to the presence of a very large fraction of myofibroblasts, already observed in low passage
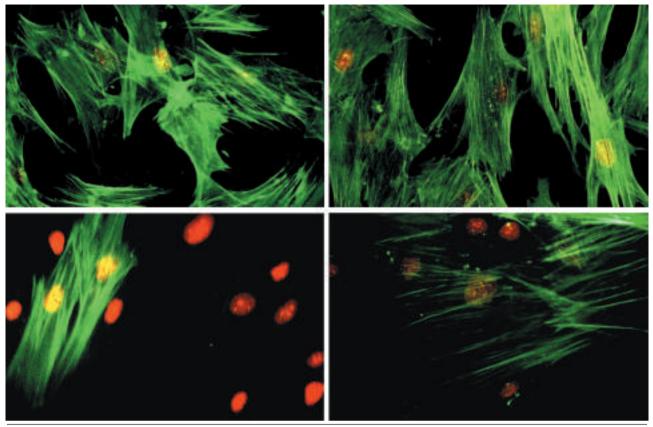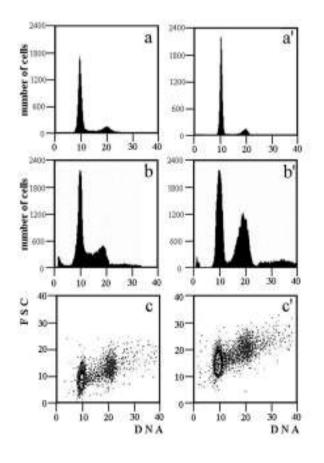
Figure 3. Fluorescence micrographs of fibroblasts (passage 7) from the unaffected skin (left column) or the nodule (right) of the FR patient. Upper row: Positivity for FITC-conjugated phalloidin, labeling fibrillar actin; Lower row: FITC-immunolabeling for smooth-muscle-specific α-actin. Nuclear DNA was counterstained with PI (magnification: ×40)

cultures. Furthermore, the present study has shown that both the hyperproliferative status and the myofibroblast phenotype became progressively more prominent in long-passage samples, in parallel with cell hypertrophy and polyploidization.

These findings suggest that fibroblast proliferation in FR nodules is probably related to a selection of a hyperproliferative myofibroblast subpopulation. A clonal selection has been supported also in skin fibroblasts from scleroderma patients (Jelaska et al.,1996). However, the factors influencing the selection and growth of myofibroblats in FR might be different from those acting in scleroderma. Scleroderma fibroblasts express increased levels of

Figure 4. DNA-content distribution histograms of cells from the unaffected skin (a,b) or the nodule (a',b') of the FR patient, after PI-staining: a,a', passage 3; b,b', passage 12. In the unaffected skin cultures, cells are diploid only, whereas in nodule cultures the fraction of cells with DNA-contents exceeding the 4C value apparently increased, with increasing passages. c,c': Dual parameter scattergrams of DNA content (after PI staining) versus low-angle scatter (forward scatter, FSC) of cells from the unaffected skin (c) or the nodule (c') of the FR patient, at passage 12: the higher FSC values indicates that, in nodule cultures, cells are larger than in controls.

trasforming growth factor β (TGFβ) receptors (Kawakami et al.,1998) and are more responsive to TGFβ than normal skin fibroblasts leading to an increased collagen synthesis (Varga and Jimenez 1996). On the contrary, the collagen synthesis is decreased in FR, while the proliferative activity is greatly enhanced. The fact that myofibroblats proliferate faster than control fibroblasts suggests that they may be more sensitive to the serum growth factors in the culture medium. Since hyperproliferation was already observed at very low (2-4) passages, it is likely that myofibroblats may express a similar response also *in vivo*, under the effect of endogenous growth-stimulating molecules (Lacour et al.,1993).

Based on these findings, we may hypothesize that the development of the typical nodules in FR could depend on the intrinsic capability of selected myofibroblats of proliferating faster than normal fibroblasts. Thus myofibroblats would increase in number and become polyploid and hypertrophic. Our electrophoretic data suggest that fibroblasts deriving from the nodule site of the FR patient synthesize slightly less collagen and non collagen proteins than do fibroblasts taken from the unaffected skin area. This appears to be in agreement with previously reported findings (Lacour et al., 1993), and could suggest that the increased fibrosis observed in nodules *in situ* could rather be dependent on a reduced degradation of the extracellular matrix components.

Acknowledgments

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