ELSEVIER

Biochimica et Biophysica Acta 1501 (2000) 51–62

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Abnormal phenotype of in vitro dermal fibroblasts from patients with pseudoxanthoma elasticum (PXE)

D. Quaglino Jr. *, F. Boraldi, D. Barbieri, A. Croce, R. Tiozzo, I. Pasquali Ronchetti

Department of Biomedical Sciences- General Pathology, University of Modena and Reggio Emilia, Via Campi 287, 41100 Modena, Italy

Received 12 October 1999; received in revised form 6 January 2000; accepted 19 January 2000

Abstract

Pseudoxanthoma elasticum (PXE) is a genetic connective tissue disease, whose gene and pathogenesis are still unknown. Dermal fibroblasts from patients affected by PXE have been compared in vitro with fibroblasts taken from sex and age-matched normal individuals. Cells were grown and investigated in monolayer, into three-dimensional collagen gels and in suspension. Compared with normal cells, PXE fibroblasts cultured in monolayer entered more rapidly within the S phase and exhibited an increased proliferation index; on the contrary, similarly to normal fibroblasts, PXE cells did not grow in suspension. Furthermore, compared with normal fibroblasts, PXE cells exhibited lower efficiency in retracting collagen type I lattices and lower adhesion properties to collagen type I and to plasma fibronectin. This behavior was associated with higher expression of integrin subunits α2, α5, αv, whereas β1 subunit as well as α2β1 and α5β1 integrin expression was lower than in controls. Compared to controls, PXE fibroblasts had higher CAM protein expression in accordance with their high tendency to form cellular aggregates, when kept in suspension. The demonstration that PXE fibroblasts have altered cell-cell and cell-matrix interactions, associated with modified proliferation capabilities, is consistent with the hypothesis that the gene responsible for PXE might have a broad regulatory role on the cellular machinery.

Keywords: Integrin; Cell cycle; Cell-matrix interactions; Connective tissue disease; Genetic disease; Pseudoxanthoma elasticum (PXE)

1. Introduction

Pseudoxanthoma elasticum (PXE) is an inherited connective tissue disorder mainly characterized by time-dependent mineralization of elastic fibers [1–3]. The most evident clinical manifestations are represented by white-yellow papules and redundant ridges in the skin, especially at the flexural areas, angioid streaks around the macula which may progress leading to retinal hemorrhages and visual loss. However, mineralization up to occlusion and hemorrhages has been described in vessels of almost all organs [4].

PXE has been recognized for decades as an elastic fiber disorder, however polymorphism, fragmentation and mineralization of the elastic component are not the only pathological manifestations in the dermis of PXE subjects. Abnormal masses of thread material containing proteoglycans and a series of

Abbreviations: CAM, cell adhesion molecules; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; PGs, proteoglycans; TCA, trichloroacetic acid

* Corresponding author. Fax: +39-59-428623;
E-mail: quaglino.daniela@unimo.it

0925-4439/00/$ – see front matter
PII: S0925-4439(00)00007-7

other matrix molecules [5–7] as well as collagen fibril abnormalities have been frequently observed in the affected dermis of these patients [6]. The recent observations that the PXE gene is localized into the region p13.1 of chromosome 16 [8,9], even though the gene and its product are still unknown, clearly indicate that elastin, whose gene is on chromosome 7 [10], is not primarily involved. Therefore, elastin mineralization as well as collagen and proteoglycan alterations may represent secondary events of a cell derangement whose comprehension may help in understanding the pathogenesis of clinical manifestations.

Up to now, very little attention has been paid to PXE fibroblasts in vivo as well as in vitro. It has been observed that in the affected dermis fibroblasts are numerous, large, polymorphic with dilated cisternae of the endoplasmic reticulum [6,11]. Moreover, tissue samples in vitro have been show to incorporate labeled amino acids into proteins with higher efficiency than normal skin [12]. When cultured in vitro, PXE dermal fibroblasts grow very actively and have been shown to have higher synthetic capabilities for collagen and elastin [12], to exhibit abnormal serine protease activity [13] and altered proteoglycan metabolism [11,14]. Actually, PXE fibroblasts produce families of proteoglycans with higher molecular weights compared to controls [11] and which differ from controls also for their sulfation characteristic [14]. Moreover, preliminary results, on in vitro cultured cells, seem to indicate that PXE fibroblasts are characterized by peculiar cytoskeletal structures, consistent with the hypothesis of altered cell-matrix interactions [15].

Since cell-matrix interactions play a crucial role in the control of gene expression, thus regulating cell survival, growth, differentiation, motility and synthetic capability [16,17], aim of the present investigation was to perform in vitro studies for a better characterization of fibroblasts from clinically affected and unaffected dermis of PXE patients, in comparison with fibroblasts derived from sex and age-matched healthy subjects, mainly focusing on cell-matrix interactions and proliferation capabilities.

2. Methods

2.1. Cells and tissues

Dermal biopsies from the neck or the armpit were obtained after informed and signed consent from five control women (age 39 ± 6 y) and from five women affected by PXE (age 43 ± 2 y). In two patients, punch biopsies were taken from both affected and unaffected areas. From each bioptic sample, fibroblast cultures were established and small fragments were fixed and embedded for electron microscopy in order to have always a morphologic assessment of the cellular and extracellular compartments in situ.

2.2. Cell cultures

Cells, unless otherwise specified, were grown in monolayer in Dulbecco's modified Eagle's medium (DMEM) plus antibiotics in 25 cm^2 flasks (Nunc, Roskilde, Denmark) and were used between the third and the eight passage.

Synchronized cells were obtained by 12 day incubation of nearly confluent cells in DMEM plus 1% FCS [18] and the G_0 phase was checked by flow cytometry.

For suspension cultures, freshly trypsinized fibroblasts were plated on a polymerized agarose gel type VII (0.9% in DMEM-F12 or DMEM) in 100 mm Petri dishes for bacteriology; 1×10^6 cells in 10 ml of DMEM-F12 or DMEM containing 15 mM HEPES pH 7.4, 1 mg/ml crystalline BSA, 25 nM sodium selenite, 5 μg/ml human transferrin, 10 μg/ml bovine insulin [19] were added to each plate.

2.3. Cell growth

In order to obtain the growth curve, 1.2×10^4 cells were cultured in monolayer into 35 mm Petri dishes (Nunc) in 2 ml DMEM plus 10% FCS. After 1, 2, 3 and 4 days of culture, cells were trypsinized, centrifuged and resuspended in a small amount of medium. Aliquots of this suspension were used to assess the cell number by mean of the Neubauer chamber.

The duplication time (DT) was calculated using the following formula:

$$DT = 48 : [(\log 2 \text{ cell number after } 48 \text{ h}) - (\log 2 \text{ cell number at time } 0)]$$

To assess thymidine incorporation, 120 000 cells were plated in 35 mm Petri dishes and cultured for 1, 2, 3 and 5 days. At each time point, 10 μl of [^{3}H]thymidine (1 mCi/ml) were added to the medium and after 3 h incubation at 37°C, the medium was removed and the monolayer washed twice with cold HBSS, 5% trichloroacetic acid (TCA) and absolute ethanol. The monolayer was air dried and then treated with 0.3 N NaOH for 10 h at room temperature. 0.5 ml of the cellular suspension was placed in scintillation vials, added with 1.5 N HCl and scintillation mixture (Ready gel, Beckman). Radioactivity was measured in a Beckman LS1701 scintillation counter. Experiments were done in triplicate.

2.4. Cell cycle

For the analysis of the cell cycle in monolayer, nearly confluent cells (3×10^5 cells in a 25 cm flask) were synchronized in G_0 by 12 days incubation of nearly confluent cells in 7 ml of DMEM plus 1% FCS, as described above [18]. After assessment of the G_0 state by flow cytometry, cells were induced to enter in the S phase by replacement of the medium with DMEM plus 20% FCS. After 4, 12, 18, 24, 30, 42 and 48 h culture, the cell cycle was assayed by incubation with 10 μM bromodeossiuridine (BrdU) for 30 min at 37°C; cells were then trypsinized, suspended in DMEM, treated with 0.5% Tween 20 (Merck) centrifuged at 2000 rpm for 5 min, resuspended in 0.5 ml 0.5% Tween 20 in PBS and in 0.5 ml 4 N HCl and incubated for 30 min at room temperature. After centrifugation, cells were suspended in 1 ml 0.1 M Borax (Riedel-de Haen), centrifuged, incubated for 1 h at 4°C in 200 μl 0.5% Tween 20 in PBS containing 5 μl anti-BrdU monoclonal antibody (Becton Dickinson, USA), centrifuged, incubated for 30 min at 4°C in 200 μl 0.5% Tween 20 in PBS and 5 μl of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulins (Becton Dickinson, USA), centrifuged, incubated for 15–30 min at 4°C in 200 μl 0.5% Tween 20 in PBS and in 200 μl propidium iodide (Sigma) and finally analyzed on a fluorescence activated cell sorter FACScan (Becton Dickinson, USA).

For the analysis of the cell cycle in suspension, cells were synchronized in G_0 by 3 day-incubation of early confluent cells (1×10^6 cells in 75 cm flask) in 15 ml of defined medium as specified above [19]. Briefly, after assessment of the G_0 state by flow cytometry cells were trypsinized and plated on a polymerized agarose gel in 100 mm Petri dishes in the presence of DMEM or DMEM-F12, 1 mg/ml crystalline BSA, 25 nM sodium selenite, 5 μg/ml human transferrin, 10 μg/ml bovine insulin, 5% FCS and 3 nM EGF [19]. After 18 and 24 h the cell cycle was assessed, as described above for cells in monolayer.

2.5. Cell-cell and cell-matrix interactions

2.5.1. Adhesion and spreading assay

Plastic Petri dishes (35 mm in diameter) uncoated or coated with collagen type I (Nunc) or plasma fibronectin (Nunc) were used. Trypsinized cells were washed in serum free DMEM, centrifuged and 1×10^5 cells in 1 ml suspension were added to each dish and allowed to attach by setting for 10 min, 20 min, 40 min, 1 h, 4 h up to 24 h at 37°C in 5% CO_2/95% air. Adhesion was evaluated as previously described [20]. Briefly, at each time considered, non adherent cells were removed by washes with PBS, whereas adherent cells were fixed with 3% paraformaldehyde for 10 min, washed with PBS twice, stained with crystal violet (0.5% in 20% methanol) for 15 min and extensively washed with distilled water. Color retained by cells was eluted by 0.1 M sodium citrate for 30 min at room temperature, read in a spectrophotometer at 540 nm, and considered proportional to the number of adherent cells. Cell spreading was evaluated with a phase contrast light microscope (Zeiss axiophot) after 4 and 24 h from seeding. All experiments were performed in triplicate.

2.5.2. Collagen gel retraction

Collagen gels were prepared by using both Vitrogen collagen solution (Celtrix) or collagen extracted from rat tail tendons in 0.1% acetic acid as previously described [20]. Collagen solutions were neutralized by adding 0.1 M NaOH and brought to the

concentration of 2 mg protein/ml. Cells were added at a concentration of 1×10^5 cells/ml. Aliquots (2 ml) of the cell/collagen mixture were placed in 35 mm diameter Petri dishes and allowed to polymerize at 37°C for 15–20 min. To create floating collagen gels, the edge of lattices was rimmed with a sterile pipette and the dishes were gently shaken. Floating gels were covered with 2 ml of DMEM with 10% fetal calf serum. Experiments were done in triplicate and the retraction was evaluated every 12 h during a 3 day period by measuring the gel area on a millimetric scale [21].

2.5.3. Expression of adhesion molecules

In order to investigate integrin expression, cells at confluence were removed from tissue culture flasks with 10 ml ethylenediaminetetraacetic acid (EDTA) (10 mM in PBS^-, without calcium and magnesium) at 37°C. EDTA was blocked by addition of the same amount of PBS with 10 μM calcium and 10 μM magnesium. Cells were washed twice with PBS^- and resuspended to 5×10^5 cells in 200 μl PBS^- and incubated for 30 min at 4°C with 5 μl of undiluted monoclonal antibodies against α1 (Chemicon, Temecula, USA), α2 (GIBCO BRL), α5 (GIBCO BRL), αv (GIBCO BRL) and β1 (GIBCO BRL) subunits of human integrin or against integrins α2β1 (Chemicon) and α5β1 (Chemicon) or against A-cell adhesion molecules (CAM) proteins (Sigma). After a rapid centrifugation cells were incubated for 30 min at 4°C with FITC-labeled anti mouse-immunoglobulins (Dako, Denmark; 1:10 diluted). Controls were established by using the secondary antibody alone or non-immune idiotypic immunoglobulins. Washed cells were resuspended in 250 μl of PBS^- and analyzed on a FACScan (Becton Dickinson, USA). Debris and dead cells were excluded by forward and side scatter gating. Ten thousand events were collected and evaluated from each cell type using Lysyl II Software.

2.6. Structure

2.6.1. Cell dimension

Cells grown for 48 h at confluence on 35 mm Petri dishes were trypsinized, centrifuged, washed with DMEM, centrifuged again and resuspended in DMEM. Glutaraldehyde was added to the final concentration of 1% and fixation was performed for 30 min. After staining with 1% toluidine blue, aliquots of cells were spread on coverslips and observed by a light microscope connected with an image analyzer equipped with a program for registration of the cell volume. About 1000 cells were examined for each control and PXE subject.

2.6.2. Cell morphology

Cells, grown on glass or on plastic surfaces coated or not with collagen or fibronectin, were: (a) fixed with 3% paraformaldehyde in PBS for 30 min at 4°C and observed either unstained or after staining with hematoxylin eosin or crystal violet, by a Zeiss axiophot optical microscope in the phase contrast mode (b) fixed with 3% paraformaldehyde in PBS for 30 min at 4°C, permeabilized by incubation for 5 min in serum-free DMEM and 1% Triton X-100, treated with FITC-labeled falloidin (2 μg/ml) for 15 min at room temperature and observed by a confocal microscope (c) fixed with 2.5% glutaraldehyde in Tyrode's solution, dehydrated, embedded in Spurr resin, sectioned and observed in a Siemens 1A and a Jeol 1200EXII transmission electron microscope.

Cells, grown into collagen gels or in suspension on agarose, were (a) observed and photographed with an Olympus inverted microscope (b) fixed in 2.5% glutaraldehyde in Tyrode's Solution, dehydrated, embedded in Spurr, sectioned and observed with a Siemens 1A and a Jeol 1200EXII transmission electron microscope.

3. Results

3.1. Cell growth

PXE and control fibroblasts were examined up to the eight passages in monolayer and exhibited good growth capabilities. Fig. 1 shows the growth curve of synchronized normal and PXE cells: fibroblasts from PXE patients grew faster between the second and the fourth day of culture, then, at confluence, all cells showed the same growing rate. Cells from unaffected areas of PXE patients behaved in between of control and diseased cells.

In accordance, the duplication time of PXE fibroblasts ($n=5$) was about 20% shorter than in controls

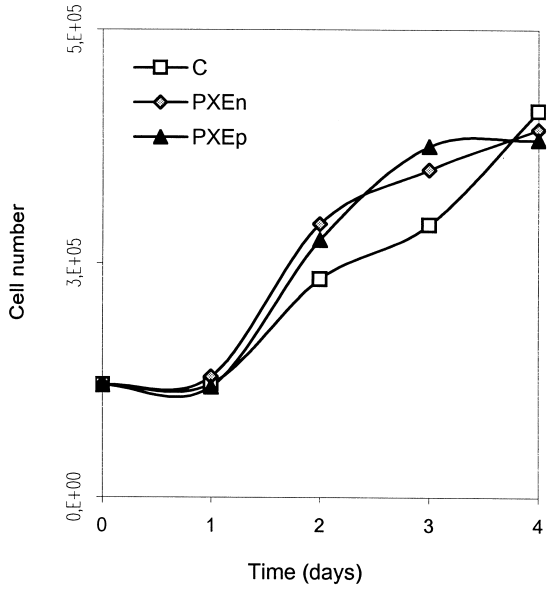

Fig. 1. Cell growth measured by means of a Neubauer chamber. Fibroblasts from PXE patients, from both clinically affected (PXEp, $n=5$) or unaffected (PXEn, $n=2$) areas, grow faster than control cells (C, $n=5$). Experiments have been performed three times in triplicate.

($n=5$) grown in parallel (30.5 vs. 38.7 h of control cells; $P<0.003$, by Student's t-test performed on results from experiments repeated three times), and cells from unaffected areas of patients had a duplication time in between.

Interestingly, after 24 h culture [^{3}H]thymidine incorporation was lower in diseased fibroblasts compared to control cells (Fig. 2). These data can be better understood from the analysis of the cell cycle between 12 and 24 h of culture. No significant differences were noted between fibroblasts from clinically affected or unaffected areas of patients.

3.2. Cell cycle

For this purpose, nearly confluent cells were synchronized by 12 days incubation in low serum medium, as described in Section 2. Four hours after addition of high serum concentration, PXE fibroblasts were still in the G_0 phase, whereas 3% of control cells were in the S phase (Fig. 3). From 12 to 24 h both control and PXE cells entered the S phase, being the number of PXE cells always higher than that of controls. After 24 h, 26% of diseased fibroblasts were in the S phase compared with the 9% of control cells (Fig. 3). Thirty hours after addition of high serum concentration, 17 and 14% of PXE and control cells respectively were in the G2/M phase (Fig. 3). After 48 h almost all cells were again in the G_0 phase (Fig. 3).

As expected for dermal fibroblasts, both control and PXE cells remained in the G_0 phase and never entered the S phase when grown in suspension (data not shown).

3.3. Adhesion

Normal dermal fibroblasts showed a discrete adhesion capability onto plastic uncoated dishes, whereas they appeared to adhere very efficiently onto plasma fibronectin and collagen type I coated dishes. By contrast, PXE fibroblasts exhibited at all times lower attachment properties to uncoated dishes ($P<0.001$), to collagen type I ($P<0.0001$) and to fibronectin ($P<0.001$) (Fig. 4). Interestingly, cells from the unaffected dermis of PXE patients exhibited a behavior that was between normal and diseased cells (Fig. 4).

3.4. Collagen gel retraction

Cell-matrix interactions were also investigated by

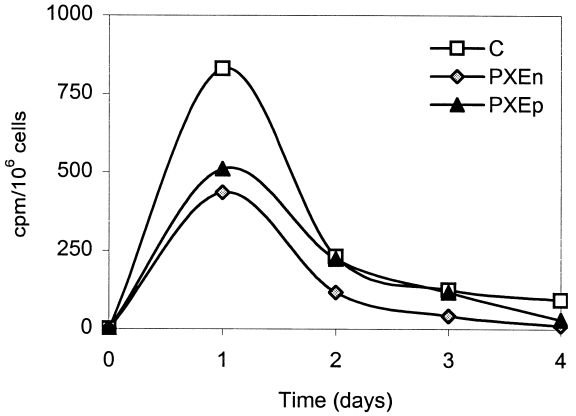

Fig. 2. [^{3}H]Thymidine incorporation of cultured dermal fibroblasts. PXE fibroblasts from both clinically affected (PXEp, $n=5$) or unaffected (PXEn, $n=2$) areas show reduced levels of incorporation compared to fibroblasts from healthy subjects (C, $n=5$), in accordance with the observation that, after the first 24 h of culture, pathologic fibroblasts already had incorporated the radioactive thymidine. Experiments have been performed three times in triplicate.

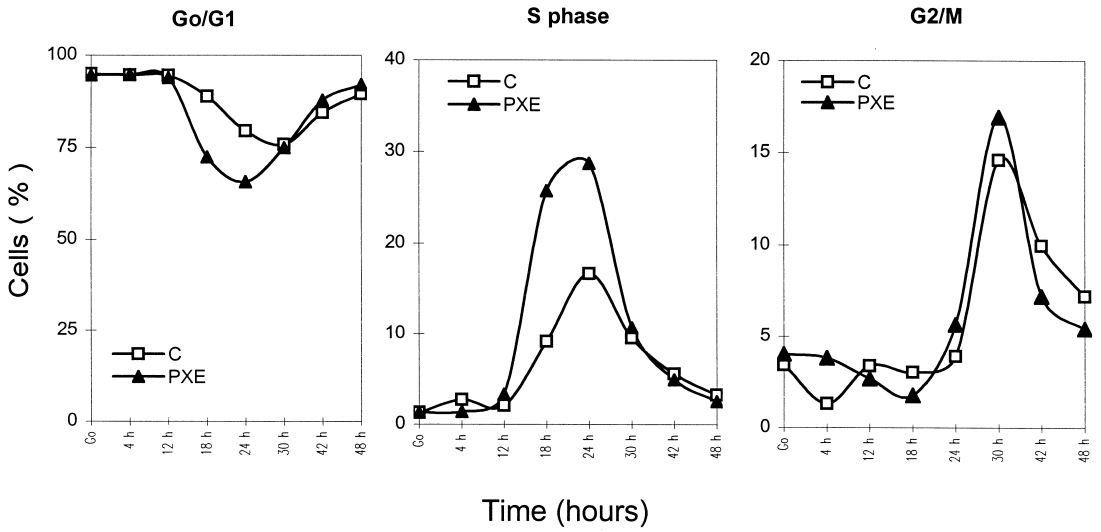

Fig. 3. Cell cycle evaluated by FACS analysis in control (C, $n=5$) and PXE (PXE, $n=5$) fibroblasts. Numerous pathologic cells enter more rapidly in the S phase, between 12 and 24 h, in agreement with data from cell growth and [^{3}H]thymidine incorporation. Experiments have been performed four times.

growing cells into floating collagen matrices made of either Vitrogen or collagen extracted from rat tail tendons and by measuring the lattice retraction with time up to 3 days. PXE fibroblasts were significantly less efficient than normal cells in retracting collagen gels at all times investigated, becoming the difference mostly evident after the first 24 h of culture (Fig. 5, $P<0.05$).

3.5. Cell-cell and cell-matrix adhesion molecules

The integrin pattern of normal and diseased fibro-

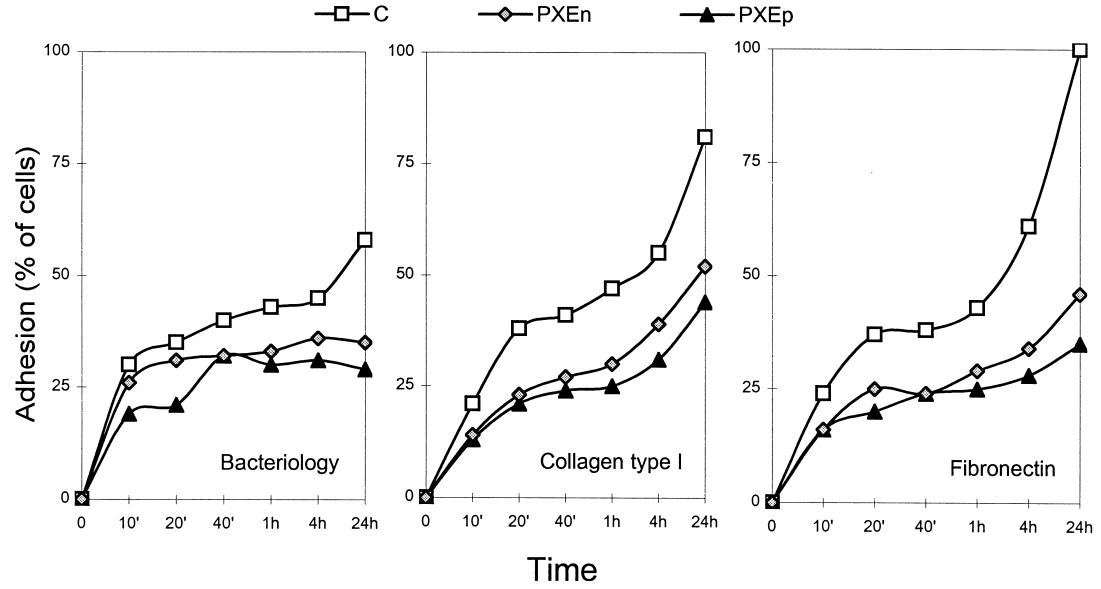

Fig. 4. Adhesion of control (C, $n=5$) and PXE fibroblasts, from both clinically affected (PXEp, $n=5$) or unaffected (PXEn, $n=2$) areas, on untreated plastic dishes for bacteriology, or collagen type I- and fibronectin-coated Petri dishes. On all substrates PXE cells adhere less efficiently compared to control fibroblasts. Data are expressed as percent of attached cells compared to the highest values of optical density obtained in control cells plated for 24 h on to fibronectin taken as 100. Experiments have been performed three times in triplicate.

blasts was investigated by FACS analysis on synchronized cells grown in monolayer at confluence. As illustrated in Fig. 6, PXE fibroblasts, compared to normal cells, exhibited a reduced expression of α1 and β1 ($P<0.05$) integrin chains and of dimers α2β1 ($P<0.05$) and α5β1. By contrast, pathologic cells showed higher expression of α_2 ($P<0.01$), α5 ($P<0.01$) and αv chains, compared to control cells.

The presence of CAM proteins was investigated by FACS analysis on synchronized cells grown in monolayer as well as in suspension. No significant differences between normal and PXE fibroblasts were seen when cells were grown in monolayer (Fig. 7); by contrast, when cells were kept in suspension, a significant increase of the CAM protein expression was observed in PXE fibroblasts compared to controls (Fig. 7, $P<0.05$).

3.6. Morphology

The mean cell volume, as measured by an image analyzer on cells detached from the growing substrate and fixed in suspension, was 789 ± 160 μm^3 and 1191 ± 183 μm^3 for control ($n=4$) and PXE dermal fibroblasts ($n=5$), respectively ($P<0.05$, by Student's *t*-test performed on results from experiments repeated four times).

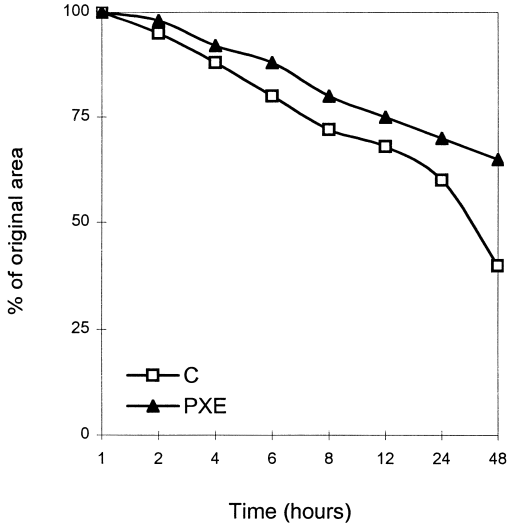

Fig. 5. Collagen gel retraction by control (C, $n=3$) and PXE ($n=5$) fibroblasts. Pathologic cells exhibited a reduced contraction of the gels suggesting the presence of altered cell-matrix interactions. Experiments have been performed two times in triplicate.

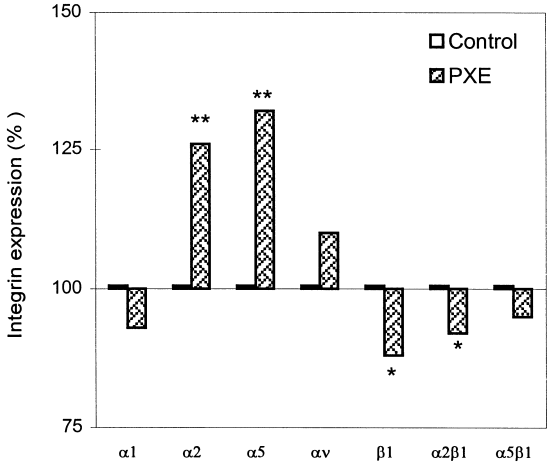

Fig. 6. Integrin expression by FACS analysis in control (C, $n=5$) and PXE ($n=5$) fibroblasts. Pathologic cells exhibited high expression of the α2, α5 and αv subunit, but low expression of the α1 and β1 subunit and of the α2β1 and α5β1 dimers. Data are represented as percentage variation of values obtained for each integrin in normal dermal fibroblasts that were always considered as 100. $*P<0.05$; $**P<0.01$ by Student's *t*-test. Experiments have been performed four times.

By light microscopy, control dermal fibroblasts, grown at confluence on glass slides in standard conditions, exhibited a typical elongated shape with frequent intercellular connections. At ultrastructural level, abundant ribosomes and a well-developed endoplasmic reticulum were observed (data not shown). Dermal fibroblasts from PXE patients appeared more polymorphous and larger than controls, with exuberant cytoplasm. By electron microscopy, PXE cells exhibited numerous mitochondria, huge and dilated cisternae of the endoplasmic reticulum (data not shown). Moreover, in long term cultures, up to 9 days, both control and PXE cells were surrounded by an extracellular matrix that was always more abundant in PXE than in control cells [11].

When seeded on bacteriological dishes normal fibroblasts adhere quite rapidly, although remaining elongated and stretched out, whereas on matrix substrates they appeared more numerous and expanded. PXE fibroblasts spread less efficiently on all tested substrates, being more globular with thin cytoplasmic projections (data not shown). After 24 h, control fibroblasts on bacteriological dishes were still elongated with thin cytoplasmic extrusions (Fig. 8A); by contrast, when seeded on collagen type I or on fibronectin they appeared well spread and had a distended

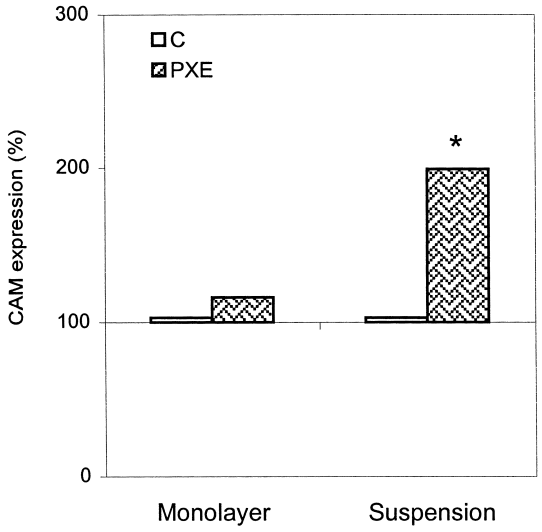

Fig. 7. CAM expression by FACS analysis on cells grown in monolayer and in suspension. A significant increase of CAM expression was observed in PXE cells ($n=4$) grown in suspension compared to control cells (C, $n=4$). Data are represented as percentage variation of values obtained in normal dermal fibroblasts that were always considered as 100. $^*P<0.05$ by Student's t-test. Experiments have been performed three times.

polyhedric shape (Fig. 8B,C). Fibroblasts from affected dermis of PXE patients spread less efficiently than control fibroblasts on all substrates (Fig. 8D,E,F). PXE cells, in facts, appeared sometimes shrunken, even on collagen type I and fibronectin substrates, and were frequently connected by long and thin cytoplasmic projections (Fig. 8E,F). The actin cytoskeleton, visualized by falloidin, was much less ordered in PXE fibroblasts compared to controls on all substrates (data not shown). Fibroblasts from unaffected areas of the dermis from PXE patients behaved in between of control and diseased cells (data not shown).

When grown into three-dimensional collagen gels control and PXE cells did not exhibit differences regarding their morphology; they were polymorphous and spread in all space directions (data not shown).

When grown in suspension on the agarose gel, both control ($n=5$) and PXE cells ($n=5$) were round and isolated one from the other up to 1 h of culture (Fig. 9A); with time, a relatively low number of cells from control subjects formed aggregates that after 24 h of culture exhibited an area of 1047 ± 392 μm^2 (Fig. 9B). By contrast, the great majority of PXE fibroblasts, after the same period of time, formed large polymorphic aggregates (1786 ± 555 μm^2; $P<0.001$ PXE vs. control, by Student's t-test on results from experiments repeated three times) (Fig. 9C). By electron microscopy, these aggregates appeared as clusters of round cells (Fig. 9D). Cells forming large aggregates were tightly packed and their plasma membranes were in close contact for long tracts (Fig. 9D, insert).

4. Discussion

PXE is a genetic connective tissue disorder, whose gene and pathogenesis are still unknown [4]. Patients are characterized by a progressive mineralization of elastic fibers, although biochemical and structural alterations of several other connective tissue components have been demonstrated in all cases investigated [5,6]. The accumulation in the extracellular space of aggregates of electrondense thread material containing glycosaminoglycans [5,7] as well as the presence of structural alterations of collagen fibrils [6] suggest a more general involvement of connective tissue metabolism, apart from elastin, in the pathogenesis of PXE [6,7].

Up to now, only few studies have been performed on in vitro cultured fibroblasts. There are reports that PXE fibroblasts in vitro exhibit high proteolytic activity, mainly due to the secretion of serine proteases into the culture medium [13]. Biochemical analysis showed that cells from the PXE-affected patients produced a proteoglycan (PG) population with stronger polyanionic properties, as well as a markedly increased amount of high hydrodynamic-size PGs [11]. In agreement with biochemical findings, the immunohistochemical analysis showed alterations affecting PG content and distribution in PXE-affected cell layers [11,14]. Furthermore, preliminary studies from our laboratory showed abnormal cytoskeletal organization in PXE fibroblasts which might be suggestive for altered cell-matrix interactions [15].

The present study was designed to characterize, from both structural and behavioral points of view, in vitro dermal fibroblasts from patients affected by Pseudoxanthoma elasticum (PXE), paying particular attention to cell-matrix interactions and to proliferation capabilities [22].

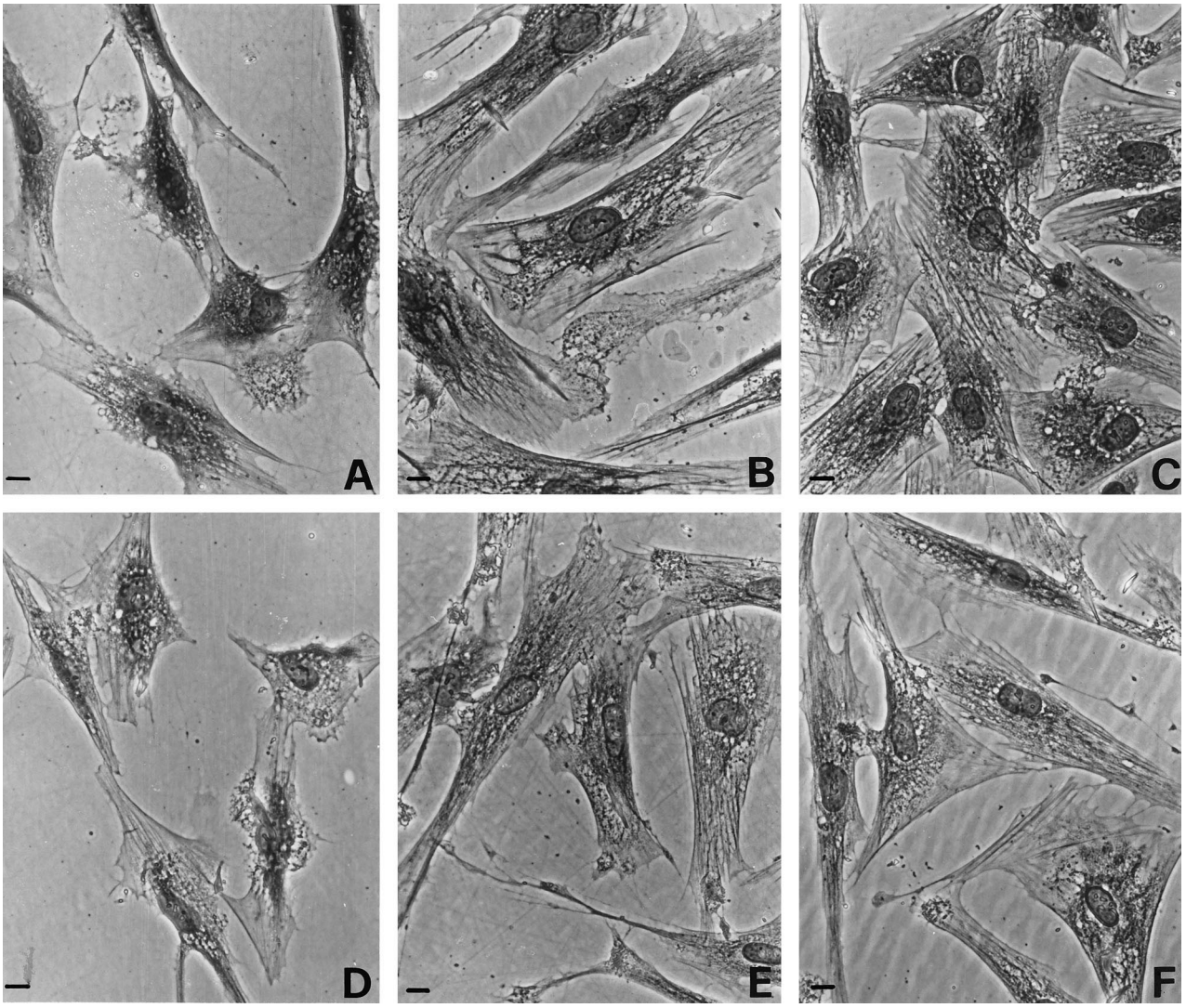

Fig. 8. Spreading capabilities of control (A,B,C) and PXE fibroblasts (D,E,F) on untreated plastic (A,D), or on collagen type I (B,E) or fribronectin-coated (C,F) Petri dishes after 24 h from seeding. PXE fibroblasts are generally less spread compared to control cells. Bar: 10 μm

Pathologic cells, grown in monolayer, were morphologically different from normal dermal fibroblasts and these differences were even more pronounced when cells were seeded on, or within, extracellular matrix substrates, such as collagen type I and fibronectin, suggesting that PXE fibroblasts might have altered cell-matrix interactions. During the first hour after seeding, as expected, normal dermal fibroblasts showed a rapid spreading on plastic, and especially on collagen type I or on fibronectin [23]. On the contrary, PXE cells remained more globular, spreading less efficiently than normal fibroblasts. Differences in cell shape were still clear after 24 h after seeding. These observations were confirmed in the adhesion tests, where, as expected [23], normal dermal fibroblasts showed high adhesiveness to collagen type I and fibronectin, the two major components of the extracellular matrix, whereas, in the same experimental conditions, PXE fibroblasts revealed significantly reduced adhesive capabilities.

These data have been further reinforced by experiments in which cells were grown into three-dimensional matrices of Vitrogen or of collagen extracted from rat tail tendons [24,25]. PXE fibroblasts exhibited a significantly lower retraction capability compared with normal cells and this may suggest scarce

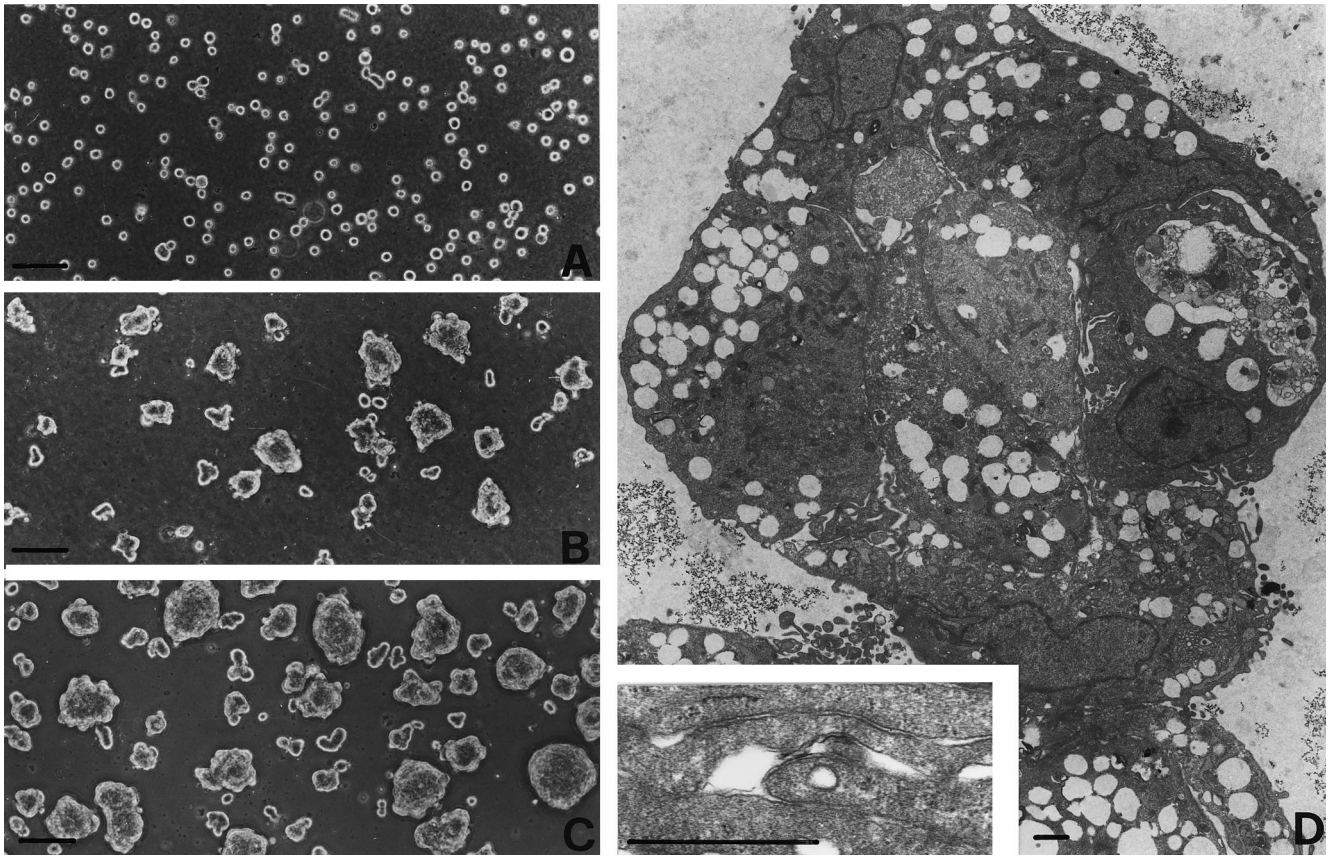

Fig. 9. Control (A) and PXE fibroblasts (B–D) grown in suspension and observed by light (A–C) and electron (D) microscopy. After 1 h from seeding on agarose type VII, control cells (A) as well as pathologic fibroblasts are round and isolated one from the other. After 24 h from seeding, pathologic cells (C) form aggregates bigger than control fibroblasts (B), in accordance with the presence of increased cell-cell interactions. At the ultrastructural level, these aggregates are comprised of cells (D) whose plasma membranes are in close apposition (D, insert). Bar: 100 μm (A–C) and 1 μm (D).

cell-collagen interactions or scarce capability of cells to exert dynamic forces [26].

The indication that cell-matrix interactions may be altered in PXE cells has been at least partially explained by FACS analysis of integrins. PXE cells exhibited a significantly lower expression of the β1 chain or of the dimers α2β1 and α5β1 compared to controls. As the β1 chain is involved in a series of integrin dimers in combination with different α chains, and dimers α2β1 and α5β1 are mainly responsible for interactions of cells with collagen and with fibronectin [27], a significant lower expression of these molecules clearly indicates that PXE cells have scarce interactions with at least two of the main extracellular components that are involved in cell attachment and may explain their scarce adhesion properties and less efficient collagen gel retraction. Interestingly, the expression of α2 and α5 integrin subunits was significantly higher in PXE fibroblasts compared with normal cells, and differences, although not significant, were also observed regarding the α1 and αv chains. These data seem to suggest a deregulation in the synthesis, maturation and/or assembly of a number of integrins in PXE fibroblasts and this point should be further investigated by considering the importance of cell-matrix interactions in cell growth, differentiation and metabolism [27,28].

Little is known concerning the factors modulating the anchorage-dependent growth of dermal fibroblasts [29]. Integrins and matrix molecules seem to play a crucial role in this context, since proliferation of normal fibroblasts can be blocked by growing the cells in suspension on agarose [19]; by contrast, a number of other cells, such as cancer cells or chon-

drocytes, may grow and differentiate in suspension [30,31]. When kept in suspension, PXE dermal cells behaved as normal dermal fibroblasts, as they did not enter the S phase. Interestingly, both normal and PXE fibroblasts, when kept in suspension, tended to form aggregates, however, diseased fibroblasts exhibited a higher tendency to aggregate compared to controls. This phenomenon can be explained by the higher expression of CAM proteins on PXE fibroblasts and is suggestive for decreased cell-matrix interactions associated with increased cell-cell adhesion properties.

When grown in monolayer, fibroblasts from PXE patients showed increased proliferation, as suggested by the decreased duplication time, the increased number of cells entering the S-phase and by lower incorporation of thymidine by PXE fibroblasts at 24 h, since at this time a great number of PXE cells was already in the S phase. It is already known that integrin-mediated cell adhesion to matrix substrates provides signals necessary for cell cycle progression and differentiation [32]. Moreover, it has been shown that the $\alpha5\beta1$ integrin modulates cell growth and regulates gene expression [33]. In particular, disruption of ligation between this integrin and fibronectin stimulates DNA synthesis [34], whereas increased expression of the $\alpha5\beta1$ integrin may reduce the mitogenic activity in fibrosarcoma cell lines [35].

As already mentioned, PXE fibroblasts, compared to control cells, have reduced expression of some integrins (namely $\beta1$, $\alpha2\beta1$ and $\alpha5\beta1$); therefore, the decreased cell-matrix interactions on synchronized cells could be related to the higher proliferation capability of PXE cells and could be a consequence of the PXE gene defect. Although performed on a limited, but clinically and genetically well defined, number of patients, these results clearly indicate that the gene responsible for PXE has to play a broad regulatory role on mesenchymal cell behavior and metabolism leading to an unbalanced production of matrix components. Therefore, elastin and the extracellular matrix in general would be dramatically involved only as a final event. Moreover, elastic fibers, because of their peculiar physico-chemical properties and slow turnover, might be affected more than other components by the alterations of the extracellular matrix homeostasis in PXE. Furthermore, as for other genetic disorders, dermal fibroblasts may represent a reliable model for investigating on the pathogenesis of connective tissue genetic disorders, however, since cells from unaffected areas of the same PXE patient behaved in between control and diseased cells, it may be hypothesized that also local factors may be involved contributing to the severity of clinical signs in PXE.

Acknowledgements

The present investigation has been performed with Telethon Grant n. E 696. Authors are indebted to Dr. Giuseppe Mori for his invaluable technical assistance.

References

[1] L. Danielsen, T. Kobayasi, H.W. Larsen, K. Midtgaard, H.E. Christensen, Acta Derm.-Venereol. 50 (1970) 355–373.
[2] F.M. Pope, J. Med. Genet. 11 (1974) 152–157.
[3] A. De Paepe, D. Viljoen, M. Matton, P. Beighton, V. Lenaerts, K. Vossaert, S. De Bie, D. Voet, J.J. De Laey, A. Kint, Am. J. Med. Genet. 38 (1991) 16–20.
[4] K.H. Neldner, Clin. Dermatol. 6 (1988) 1–159.
[5] A. Martinez-Hernandez, W.E. Huffer, Lab. Invest. 31 (1974) 181–186.
[6] I. Pasquali-Ronchetti, D. Volpin, M. Baccarani-Contri, I. Castellani, A. Peserico, Dermatologica 163 (1981) 307–325.
[7] I. Pasquali-Ronchetti, M. Baccarani-Contri, C. Pincelli, G.M. Bertazzoni, Arch. Dermatol. Res. 278 (1986) 386–392.
[8] B. Struk, K.H. Neldner, V.S. Rao, P. St-Jean, K. Lindpaintner, Hum. Mol. Genet. 6 (1997) 1823–1828.
[9] S. van-Soest, J. Swart, N. Tijmes, L.A. Sandkuijl, J. Rommers, A.A. Bergen, Genome Res. 7 (1997) 830–834.
[10] M.J. Fazio, M.G. Mattei, E. Passage, M.L. Chu, D. Black, E. Solomon, J.M. Davidson, J. Uitto, Am. J. Hum. Genet. 48 (1991) 696–703.
[11] R. Tiozzo-Costa, M. Baccarani-Contri, M.R. Cingi, I. Pasquali-Ronchetti, R. Salvini, S. Rindi, G. De-Luca, Coll. Relat. Res. 8 (1988) 49–64.
[12] N. Blumenkrantz, L. Danielsen, G. Asboe-Hansen, Acta Dermatovenerol. 53 (1973) 435–438.
[13] S.G. Gordon, M. Overland, J. Foley, Connect. Tissue Res. 6 (1978) 61–68.
[14] A. Passi, R. Albertini, M. Baccarani-Contri, G. de-Luca, A. de-Paepe, G. Pallavicini, I. Pasquali-Ronchetti, R. Tiozzo, Cell Biochem. Funct. 14 (1996) 111–120.
[15] M. Baccarani-Contri, R. Tiozzo, M.A. Croce, T. Andreoli, A. De-Paepe, Cytotechnology 11 (Suppl.1) (1993) S112–S114.
[16] S. Dedhar, Curr. Opin. Hematol. 6 (1999) 37–43.

[17] E.H. Danen, R.M. Lafrenie, S. Miyamoto, K.M. Yamada, Cell Adhes. Commun. 6 (1998) 17–24.
[18] T. Ashihara, R. Baserga, Methods Enzymol. 58 (1979) 248–262.
[19] T.M. Guadagno, R.K. Assoian, J. Cell Biol. 115 (1991) 1419–1425.
[20] D.Jr. Quaglino, G. Bergamini, M.A. Croce, F. Boraldi, D. Barbieri, A. Caroli, A. Marcuzzi, R. Tiozzo, I. Pasquali-Ronchetti, J. Cell Physiol. 173 (1997) 415–422.
[21] S.A. Santoro, M.M. Zutter, J.E. Wu, W.D. Staatz, E.U. Saelman, P.J. Keely, Methods Enzymol. 245 (1994) 147–183.
[22] S. Nakagawa, P. Paswelek, F. Grinnell, Exp. Cell Res. 182 (1989) 572–582.
[23] C.E. Schmidt, T. Chen, D.A. Lauffenburger, Biophys. J. 67 (1994) 461–474.
[24] C. Guidry, F. Grinnell, Coll. Relat. Res. 6 (1987) 515–529.
[25] J.P. Heath, L.D. Paechey, Cell Motil. Cytoskeleton 14 (1989) 382–392.
[26] M. Eastwood, R. Porter, U. Khan, G. McGrouther, R. Brown, J. Cell Physiol. 166 (1996) 33–42.
[27] J. Labat-Robert, Pathol. Biol. Paris 40 (1992) 883–888.
[28] F.T. Bosman, Histochem. J. 25 (1993) 469–477.
[29] Y. Shiba, T. Ohshima, M. Sato, Biotechnol. Bioeng. 57 (1998) 583–589.
[30] H. Inoue, J. Kondo, T. Koike, C. Shukunami, Y. Hiraki, Biochem. Biophys. Res. Commun. 241 (1997) 395–400.
[31] R.W. Tucker, K.K. Sanford, S.L. Handleman, G.M. Jones, Cancer Res. 37 (1977) 1571–1579.
[32] A.J. Garcia, M.D. Vega, D. Boettiger, Mol. Biol. Cell 10 (1999) 785–798.
[33] J.A. Varner, D.A. Emerson, R.L. Juliano, Mol. Biol. Cell 6 (1995) 725–740.
[34] J. Gong, T.C. Ko, M.G. Brattain, J. Biol. Chem. 273 (1998) 1662–1669.
[35] D. Wang, T.M. Birkenmeier, J. Yang, S. Venkateswarlu, L. Humphrey, M.G. Brattain, L. Sun, J. Cell Physiol. 164 (1995) 499–508.