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Immunocytochemical characterisation of rabbit and mouse embryonic fibroblasts

Vratislav Horák[a]*, Jacques-Edmond Fléchon[b]

[a] Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, CZ-277 21 Liběchov, Czech Republic
[b] Inra, Biologie Cellulaire et Moléculaire, 78352 Jouy-en-Josas cedex, France

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Abstract — By using indirect immunofluorescence we demonstrated the localisation of extracellular matrix (ECM) proteins (laminin - LAM, collagen IV - COL IV, fibronectin - FN) and the basic fibroblast growth factor (bFGF) in rabbit and mouse primary embryonic fibroblasts (PEF). Proliferating mitotically arrested PEF (by mitomycin C) were compared in both species. The stability of protein expression was ascertained during the first five successive passages. In addition, STO cells (i.e. permanent line of irradiated mouse fibroblasts) were similarly analysed. Rabbit PEF showed very high extracellular staining for FN and a negligible cytoplasmic positivity for LAM and COL IV. A totally reversed staining pattern for ECM proteins was found in mouse PEF. A dense cytoplasmic granulation (concentrated around the nucleus) was revealed for LAM and COL IV and almost no reaction for FN. The staining patterns were very stable at the culture conditions we applied. They were maintained during the first five successive passages in proliferating as well as non-proliferating mouse and rabbit PEF and were independent of cell concentration (individually dispersed cells versus cells in a confluent layer). STO cells showed the same staining for ECM proteins as the mouse PEF, thus confirming their origin from the same animal species. Light granular staining for bFGF was found in the cytoplasm of proliferating and mitotically arrested rabbit and mouse PEF and STO cells. The differences in expression of ECM proteins between the rabbit and mouse PEF, as well as the synthesis of bFGF, should be taken into consideration when these cells are used in vitro as a feeder layer for various cells (e.g. embryonic stem cells). © Inra/Elsevier, Paris.

embryonic fibroblast / extracellular matrix / basic FGF

Résumé — Caractérisation immunocytochimique de fibroblastes embryonnaires de lapin et de souris. Nous avons démontré par immunofluorescence la localisation de protéines de la matrice extracellulaire (MEC), lamine (LAM), collagène IV (col IV), fibronectine (FN) et du facteur de croissance fibroblastique basique (bFGF) dans des fibroblastes embryonnaires primaires (PEF). Des PEF en prolifération ou au cycle mitotique bloqué par la mytomycine C ont été comparées dans les deux espèces. La stabilité de l'expression des protéines a été contrôlée pendant cinq passages successifs.

* Correspondence and reprints
E-mail: horakv@iapg.cas.cz

De plus, des cellules STO (c'est-à-dire une lignée permanente de fibroblastes murins irradiés) ont été analysées. Les PEF de lapin montrent une très forte coloration extracellulaire de la FN et une positivité cytoplasmique négligeable pour LAM et col IV. Un type de coloration complètement inversé pour les protéines de la MEC a été trouvé dans les PEF murins. LAM et col IV sont localisés dans des granulations cytoplasmiques (concentrées autour du noyau) alors que la FN n'est pratiquement pas détectée. Ce type de coloration est stable dans les conditions de culture utilisées : il est maintenu pendant les cinq premiers passages dans les PEF prolifératifs ou non de souris et de lapin et indépendant de la concentration cellulaire (cellules dispersées ou confluantes). Les cellules STO montrent la même coloration pour les protéines de la MEC que les PEF murins, ce qui confirme leur origine spécifique commune. Une fine coloration granulaire pour le bFGF est présente dans le cytoplasme des PEF de lapin et de souris et des cellules STO, proliférantes ou non. Les différences d'expression des protéines de la MEC entre les PEF des deux espèces et la synthèses de bFGF sont des éléments à considérer quand ces cellules sont utilisées in vitro comme assise support pour la culture de cellules diverses (par exemple des cellules souches embryonnaires). © Inra/Elsevier, Paris.

fibroblaste embryonnaire / matrice extracellulaire / FGF basique

1. INTRODUCTION

Embryonic stem (ES) cells are undifferentiated, totipotent cells which can be derived in vitro from the preimplantation embryos [15, 30]. Mouse primary embryonic fibroblasts (PEF), mitotically arrested by treatment with mitomycin C, were suggested [44] as feeder cells for the isolation and further culture of ES cells. Numerous mouse ES cell lines were successfully established using such feeder layers (e.g. [10, 14, 43, 62]). Attempts were made recently to apply ES cell technology to other mammalian species. Thus, a feeder layer of mouse PEF was also used for other species such as the pig [18] and monkey [57]. It was ascertained that mouse PEF produce leukaemia inhibitory factor (LIF) also called the differentiation inhibiting activity (DIA), a cytokine which plays a key role in the maintenance of the totipotent character of ES cells in vitro by preventing their spontaneous differentiation [38, 39, 51, 61]. STO cells, mouse irradiated fibroblasts, are also feeder cells utilised for ES cell isolation in the mouse [4, 44], cow [3] and pig [16, 56]. As in the mouse PEF, the STO cells also synthesise LIF [61], but expression of other proteins has not yet been studied. In some cases, uterine fibroblasts and/or species specific PEF were chosen as feeder cells in attempts of for porcine [53], mink [54] and rat ES cell isolation [24]. It is, however, still necessary to clarify which factors or proteins synthesised by various feeder cells are responsible for their more or less successful application to ES cell establishment since precise characterisation of feeder cells is generally lacking.

As previously shown in various classes of vertebrates, the extracellular matrix (ECM) proteins differently influence cell behaviour. A primary function of fibronectin (FN) is to provide a substrate for cell adhesion and migration [13, 23]. Its expression during embryogenesis is associated with morphogenetic events such as migration of the parietal endoderm along the trophectoderm and gastrulation [12, 32, 42, 47, 60].

Laminin (LAM) is a major structural component of all basement membranes [58]. It participates in a broad range of biological activities, including cell adhesion and migration, stimulation of cellular proliferation and differentiation and promotion of neurite outgrowth [6, 26, 31, 41]. LAM is involved in early developmental events such as mesoderm formation [33] and cell polarisation

[25]. In vitro, both FN and LAM modulate attachment and migration of mesodermal cells [27] and neural crest cells [20]. Using myogenic cells, LAM and FN showed an opposite effect on cultured cells. LAM induced their differentiation while FN promoted cell proliferation [59].

Collagens (and other components of ECM such as vitronectin and tenascin) also regulate cell differentiation [2]. In vitro, collagen (type I - COL I and type IV - COL IV) positively influenced keratinocyte attachment and proliferation [8], COL I promoted mesenchymal cell differentiation into osteoblasts [48] and induced polarisation and casein secretion in mammary epithelial cells [9, 29]. ECM components are also necessary for spreading, polarisation and specific secretory activity in vitro in the case of pig trophectoderm cell lines [28]. Another cell line of pig trophectoderm needs coculture with feeder cells which produce ECM components [17].

Growth factors are another kind of biologically active substance which influence cell proliferation and differentiation in vitro as well as developmental events in embryos, including morphogenic movements and cell diversification. Their effects are rather complex and dependent on target cells. Unknown soluble factors released from intestinal fibroblasts can modulate the proliferation and secretion of endocrine intestinal cells [40]. Some members of the heparin-binding growth factor (especially bFGF) and TGF-β (activin A, TGF-β_1, TGF-β_2) families have been found to possess similar mesoderm-inducing activity. On the contrary, bFGF showed a mitogenic effect on pluripotent neural crest cells whereas TGF-β_1 acted as an antimitotic signal [19, 21, 34, 36, 46, 49, 50].

In light of the above-mentioned results, it is necessary to take into consideration what ECM proteins and growth factors are produced by cells used for preparation of a feeder layer. Such proteins could influence growth and differentiation of cells cultured on the feeder layer. There is, however, no systematic knowledge available in this field. To fulfil the missing information, at least partially, we studied the expression and localisation of several ECM proteins (FN, LAM and COL IV) and bFGF in the rabbit and mouse PEF (during the first five passages after their isolation) as well as in STO cells using indirect immunofluorescence. Both proliferating and mitotically arrested cells were characterised.

2. MATERIALS AND METHODS

2.1. Culture of rabbit and mouse PEF

PEF were isolated from rabbit (New Zealand White) and mouse (ICR line) embryos on the 17th and 12th day of gestation, respectively, according to the technique of Robertson [44], which was modified as follows. All embryos after decapitation and removal of the soft visceral organs were collected together, cut into small pieces and treated with 0.1 % trypsin-EDTA (1 mL per embryo) at 37 °C for 15 min. Then, culture medium for PEF (Dulbecco's modified Eagle's medium – Gibco, supplemented with 10 % of foetal calf serum, penicillin, streptomycin, 2-mercaptoethanol, nucleoside mixture and non-essential amino acids) were added (2 mL per embryo) and the embryo pieces were repeatedly passed through a 10-mL pipette to release individual cells from the embryonic tissues. The arising cell suspension was filtered through a sterile nylon membrane and centrifuged at 1 000 rpm for 5 min. The supernatant was removed and isolated cells were plated on 100-mm non-gelatinised Petri dishes and incubated for a short time (37 °C, 20 min) to ensure adhesion of PEF. The culture medium was then changed and cells were cultured for the next 4 to 5 days at 37 °C in humidified 5 % CO_2. The medium was changed every 2 days. After this time, the PEF formed an almost confluent cell layer. They were treated with 0.1 % trypsin-EDTA for 5 min at 37 °C, centrifuged at 1 000 rpm for 5 min, frozen at an approximate concentration of 5×10^6 cells·mL^{-1} of the culture medium with 10 % DMSO at –80 °C overnight and stored in liquid nitrogen until use.

For the experiments, proliferating and mitotically arrested PEF were compared in both animal species during five successive passages. Round

coverslips (12 mm in diameter) were placed in 4-well plates (Nunc) and coated with 0.1 % porcine gelatin. Stored PEF were thawed and plated into wells at low (2×10^3 cells/coverslip) and high (1×10^5 cells/coverslip) cell densities. Such cell concentrations resulted in individually dispersed PEF and a confluent cell layer during the next cultivation, respectively. The culture medium was changed after 20 min at 37 °C to remove dead PEF and other cell types which could be present in the suspension together with PEF. Two days after plating, part of the coverslips with PEF was treated with mitomycin C (10 $\mu g \cdot mL^{-1}$) for 4 h at 37 °C to arrest cell proliferation. Thereafter, PEF were thoroughly washed (five times) with PBS, cultured for another 2 or 7 days upon which they were used for indirect immunofluorescence. The remaining PEF, after thawing, were plated on gelatinised 100-mm Petri dishes (6×10^5 cells/Petri dish) and passaged every 4 to 5 days (before almost reaching a confluent cell layer) until the fifth passage. PEF from individual passages were trypsinised, plated on coverslips and treated as described earlier.

2.2. Indirect immunofluorescence

Expression and localisation of ECM proteins were demonstrated by indirect immunofluorescence with anti-FN mouse monoclonal antibodies (a gift from Dr I. Virtanen, University of Helsinki, Finland), anti-LAM and anti-COL IV rabbit polyclonal antibodies (Pasteur Institute, Lyon, France). Rabbit anti-bFGF polyclonal antibodies were kindly provided by Professor D. Schams (Technische Universität München, Freising, Germany). The isolated rabbit and mouse PEF were tested for cell type purity using mouse monoclonal antibodies against vimentin, cytokeratin 18 (both kindly provided by Dr I. Virtanen, University of Helsinki, Finland), neurofilament 68 kDa (Boehringer Mannheim, Germany) and myosin heavy chains (MF-20 antibody kindly provided by Dr A. Wobus, IPK, Gatersleben, Germany; [5]) as specific markers of fibroblasts, epithelial, neuronal and myogenic cells, respectively.

Rabbit and mouse PEF and STO cells growing on coverslips were washed with Ca, Mg-PBS (PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) for 3 min. Fixation with absolute ethanol for 15 min on ice was used for all the antibodies with the exception of anti-LAM and anti-bFGF. Non-specific immunostaining was inhibited with 10 % goat serum in PBS for 1 h. An incubation with primary antibodies followed directly (without washing) at +4 °C overnight. Thereafter, the bound primary antibody was revealed with the secondary goat anti-mouse IgG/FITC antibody (Sigma, Deisenhofen, Germany; diluted 1:200) or goat anti-rabbit IgG/FITC antibody (Sanofi Diagnostic Pasteur, Marnes-la-Coquette, France; diluted 1:400) applied for 1 h at room temperature. All primary and secondary antibodies were diluted with 10 % goat serum in PBS. Cell nuclei were counterstained with propidium iodide. Finally, the coverslips with immunostained cells were mounted in Mowiol with propyl gallate and analysed by fluorescence microscopy. Washing (0.2 % BSA in PBS, three times for 5 min each) was inserted between each of the individual steps of immunostaining. For LAM and bFGF demonstration, cells were fixed with 3 % paraformaldehyde in microtubule stabilising buffer (MSB), pH 7.0 [11] for 15 min on ice and then treated in 0.1 M glycine in PBS (twice for 5 min each) for quenching. Saponin at a 0.05 % final concentration was added to all the solutions starting with 10 % goat serum to permeabilise cell membranes after paraformaldehyde fixation. Since the FITC-labelled secondary antibody did not give clear staining in the case of bFGF localisation, Cy3-labelled goat anti-rabbit antibodies (Jackson Immunoresearch Laboratory, West Grove, Pennsylvania, USA; diluted 1:1 000) with a higher sensitivity were applied. In controls, the primary antibody was replaced with 10 % goat serum in PBS.

3. RESULTS

3.1. Characterisation of rabbit and mouse PEF

For isolation of PEF according to Robertson [44], the cell suspension obtained by trypsinisation of embryos is incubated for 24 h and only thereafter is the culture medium changed. Such incubation is too long and allows adhesion of various cell types in addition to PEF. By applying this method to rabbit and mouse embryos, we obtained highly heterogeneous cell populations which included fibroblasts as well as epithelial, neuronal, smooth and skeletal muscle cells as demonstrated by indirect immunofluorescence with antibodies against specific marker proteins.

Fibroblasts generally adhere more rapidly to the culture plate surface than other cell types, thus making it possible to isolate them in a specifically high purity from heterogeneous populations. For this reason, we utilised a short incubation of only 20 min after cell plating on Petri dishes for PEF isolation as well as after thawing of stored PEF for experiments before changing the culture medium. This procedure ensured that isolated cells were almost exclusively PEF. Neither muscle cells (showing fibrillar immunostaining for myosin heavy chain) nor neuronal cells (with fibrillar positivity for neurofilaments 68 kDa) were found among mouse and rabbit cells isolated by our modified method. Epithelial cells with intense staining for cytokeratin 18 very rarely found (less than 0.5 % of all cells). Thus, isolated cell populations were composed almost exclusively of PEF was inferred from a very strong fibrillar cytoplasmic labelling of vimentin and cell morphology (*figure 1*). Similar staining patterns for vimentin were observed in both rabbit and mouse PEF as well as in STO cells.

3.2. Immunocytochemical localisation of ECM proteins and bFGF

Rabbit and mouse PEF differed clearly in the expression of ECM proteins as demonstrated by indirect immunofluorescence (*table I*). All rabbit PEF showed an intense staining for FN localised outside of the cells in the form of ECM. For other ECM studied proteins (LAM and COL IV), very light granular positivity was found in the cytoplasm (about 30 % of all cells) or the cells were totally negative (*figure 2*). On the contrary, an intense granular staining for LAM (usually concentrated around the nucleus) and for COL IV (localised outside of the cells as ECM) but almost no reactivity for FN (less than 1 % of cells with light positivity only) were observed in all mouse PEF (*figure 3*). These staining patterns were very stable in our culture conditions. They were maintained during the first five successive passages after their isolation from embryos. They were independent on cell concentrations so that no visible differences

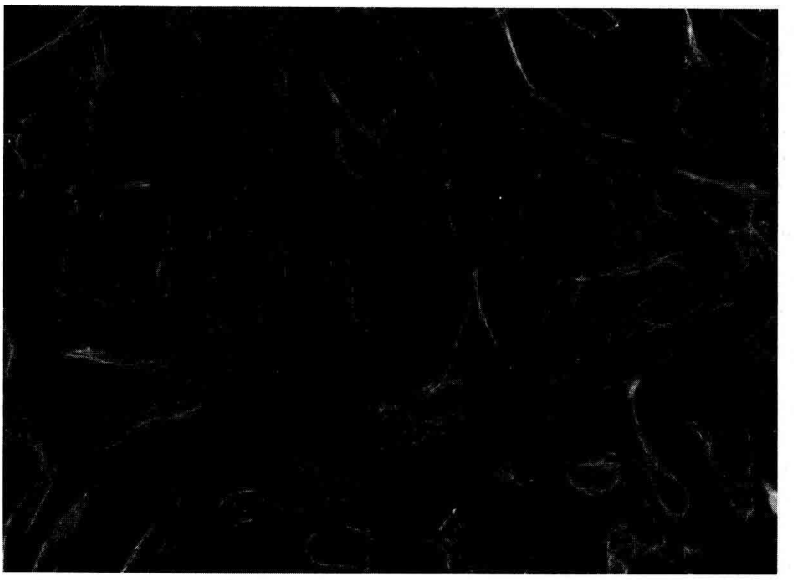

Figure 1. Fibrillar staining for vimentin was observed in the cytoplasm of isolated rabbit primary embryonic fibroblasts (PEF) (× 150). The same staining pattern was found in the mouse PEF (not shown).

Table I. Characterisation of the rabbit and mouse primary embryonic fibroblasts by indirect immunofluorescence.

Protein	Rabbit PEF	Mouse PEF
Fibronectin	++ / +++ (in form of ECM)	– (less than 1 % of cells +)
Collagen IV	– / +	++ / +++ (cytoplasmic granulation and ECM)
Laminin	– / +	++ / +++ (cytoplasmic granulation)
Vimentin	+++ (cytoplasmic fibrils)	+++ (cytoplasmic fibrils)
Cytokeratin 18	–	–
NF-68 kDa	–	–
MHC	–	–
bFGF	++ (cytoplasmic granulation)	++ (cytoplasmic granulation)

Staining intensity is graded from negative (–) to highly positive (+++). PEF: primary embryonic fibroblasts; ECM: extracellular matrix; NF: neurofilaments; MHC: myosin heavy chains; bFGF: basic fibroblast growth factor.

were detected comparing individually dispersed cells versus cells in a confluent layer. However, a higher positivity was generally observed in non-proliferating (mitomycin C-treated) PEF of both species in comparison with the proliferating PEF (*figure 4*). Despite this increase, the differences between the rabbit and mouse PEF in ECM protein expression described earlier were still found in the mitotically arrested PEF. STO cells showed the same expression and localisation of ECM proteins as the mouse PEF, thus confirming their common animal species origin.

Clear expression of bFGF was ascertained using rabbit anti-bFGF antibody and Cy3-labelled goat anti-rabbit IgG. Granulation was regularly dispersed in the whole cytoplasm of proliferating (*figure 5A*) and non-proliferating rabbit PEF. Mouse PEF (*figure 5B*) and STO cells showed a rather finer cytoplasmic granulation which was concentrated around the nucleus in some cells. This perinuclear granulation was more pronounced in non-proliferating cells (*figure 5C*) suggesting its relation to the endoplasmic reticulum. Various cell concentrations did not change the bFGF staining patterns.

4. DISCUSSION

Previous studies identified changes in various cell types in the production of ECM proteins during serial passages in culture. Increased expression in COL IV and LAM but stable COL I production was demonstrated in glomerular mesangial cells starting from the eleventh passage [45]. On the contrary, a decrease in COL I and III expression was noted with serial passages in human skin fibroblasts [55]. Using our in vitro model we did not observe any visible differences in staining intensities with antibodies for LAM, COL IV and FN during the first five passages. Thus, expression of these ECM proteins (as well as of bFGF) seems to be stable in the rabbit and mouse PEF, at least during several early passages without any effect of cell 'ageing' in culture.

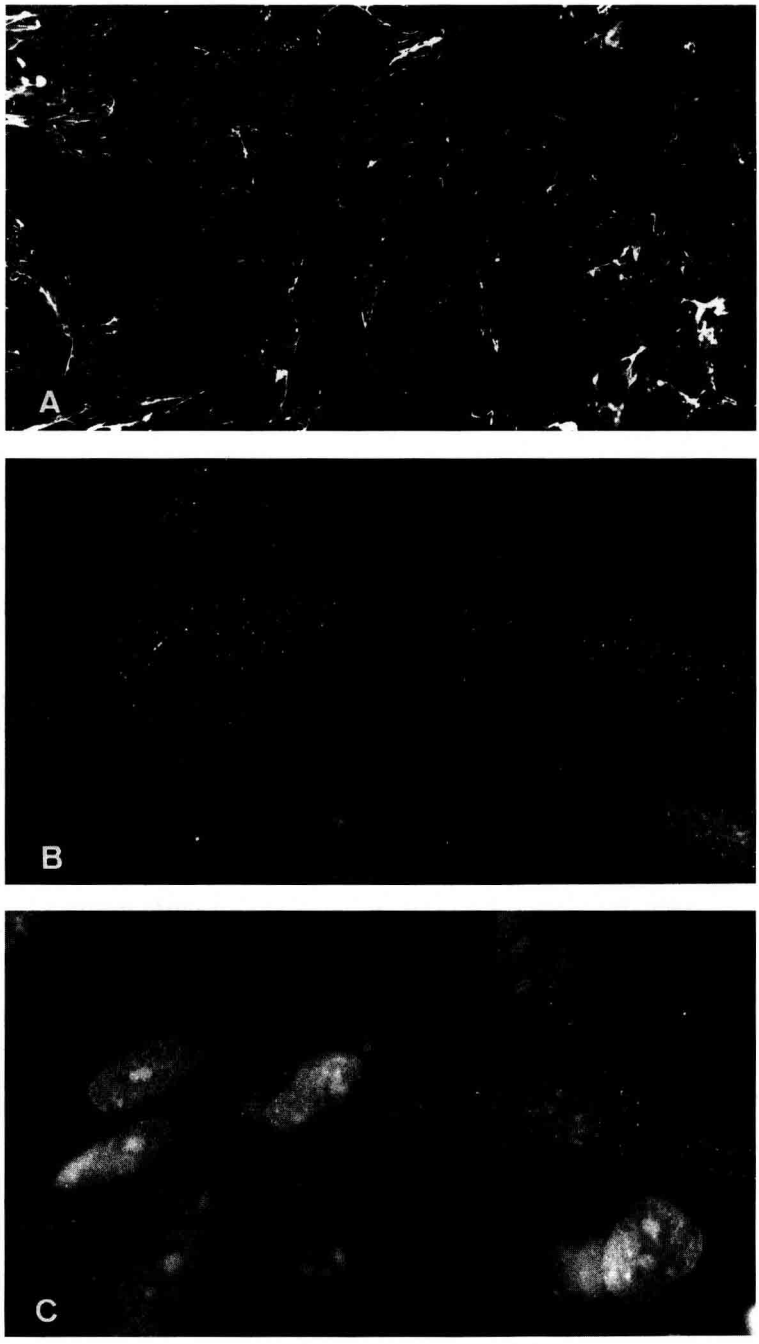

Figure 2. Immunocytochemical localisation of extracellular matrix (ECM) proteins – fibronectin (**A**), laminin (**B**) and collagen IV (**C**) – in proliferating rabbit primary embryonic fibroblasts (PEF) (× 320). Nuclei are counterstained with propidium iodide (**C**).

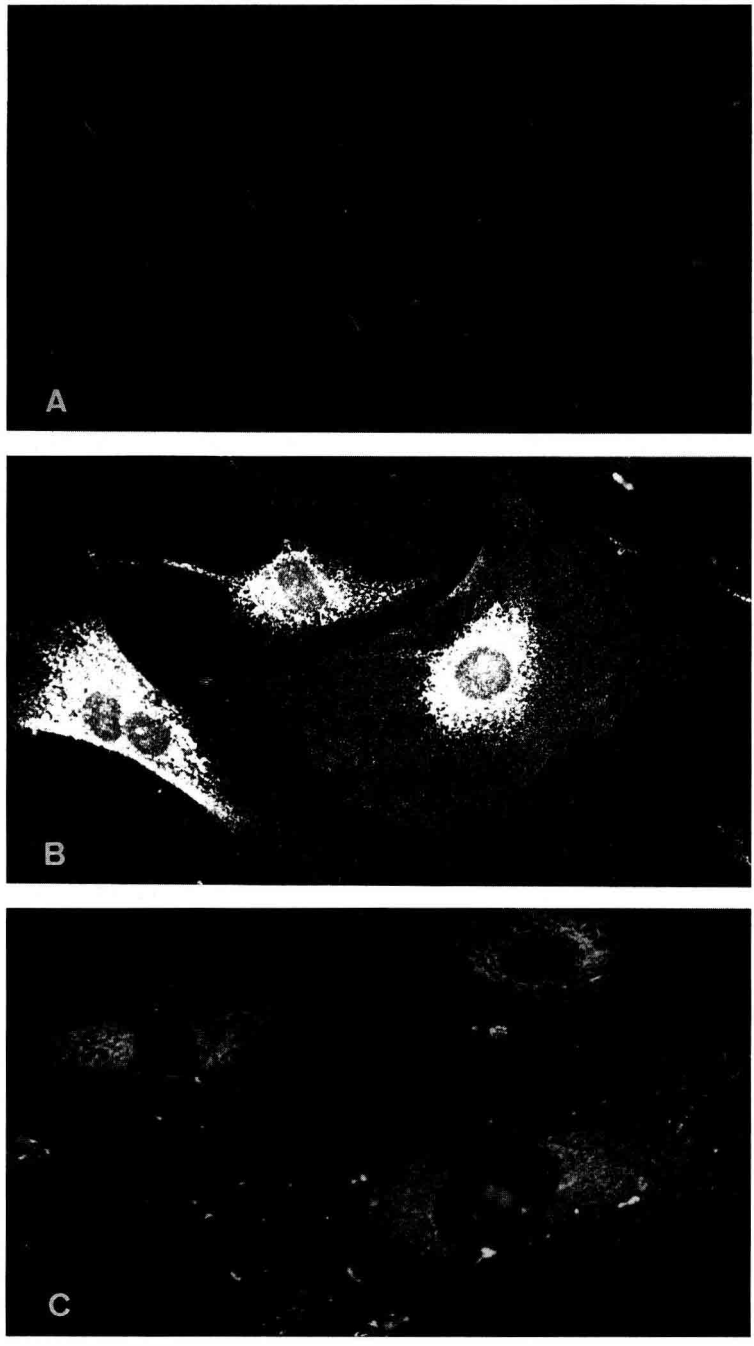

Figure 3. Immunocytochemical localisation of extracellular matrix (ECM) proteins – fibronectin (**A**), laminin (**B**) and collagen IV (**C**) – in proliferating mouse primary embryonic fibroblasts (PEF) (× 250). Compare the staining patterns with those for rabbit PEF (see *figure 2*).

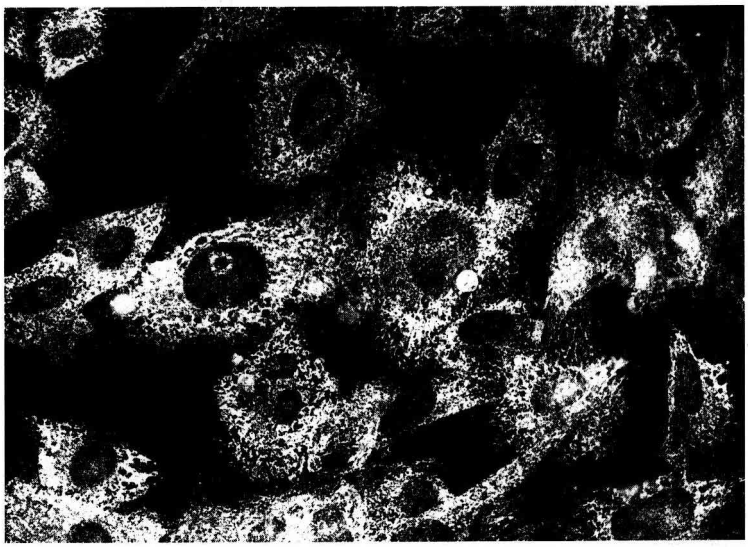

Figure 4. Expression of extracellular matrix (ECM) proteins was generally increased in non-proliferating cells as shown for laminin in mouse primary embryonic fibroblasts (PEF) (× 250). Compare the staining with that in *figure 3B*.

When culturing cells on a feeder layer it would be desirable to know which proteins are produced by feeder cells since various ECM proteins and growth factors influence differently cell proliferation and differentiation (see Introduction). Behaviour of rat blastocysts on feeders composed of mitomycin C-treated STO cells, mouse PEF, rat PEF or rat uterine epithelia cells (RUC) was tested recently [37]. Hatching and attachment of the blastocysts were similar on each feeder. However, differentiation of the inner cell mass (ICM) proceeded more rapidly on the STO and mouse PEF feeders than on the rat PEF and was highly depressed on the RUC feeder. An attempt was also made to isolate rat ES-like cells from ICM giving the best results on the RUC feeder. The observed differences were clearly related to various feeders but their characterisation from the viewpoint of protein expression or secretion was not carried out.

We found that by using indirect immunofluorescence, expression of main ECM proteins differed in the PEF of two mammalian species. Rabbit PEF showed a very high level of FN and a negligible positivity for LAM and COL IV, whereas mouse PEF revealed a high concentration of LAM and COL IV, and FN was nearly not detected. STO cells demonstrated the same staining patterns for the ECM proteins as the mouse PEF confirming a common origin of species. Rabbit and mouse PEF and STO cells treated by mitomycin C, a procedure applied to arrest mitotic division of cells used for feeder layer preparation, showed generally higher positivity for all ECM proteins studied in comparison with proliferating cells. However, the differences in ECM expression ascertained between rabbit and mouse PEF were still clearly maintained. Using these cells as feeders for ES cells (or for other cells) these differences in ECM protein expression should be taken into consideration in order to induce either proliferation or differentiation of cultured cells.

Integrins are specific cell surface ECM protein receptors (for review see e.g. [22]).

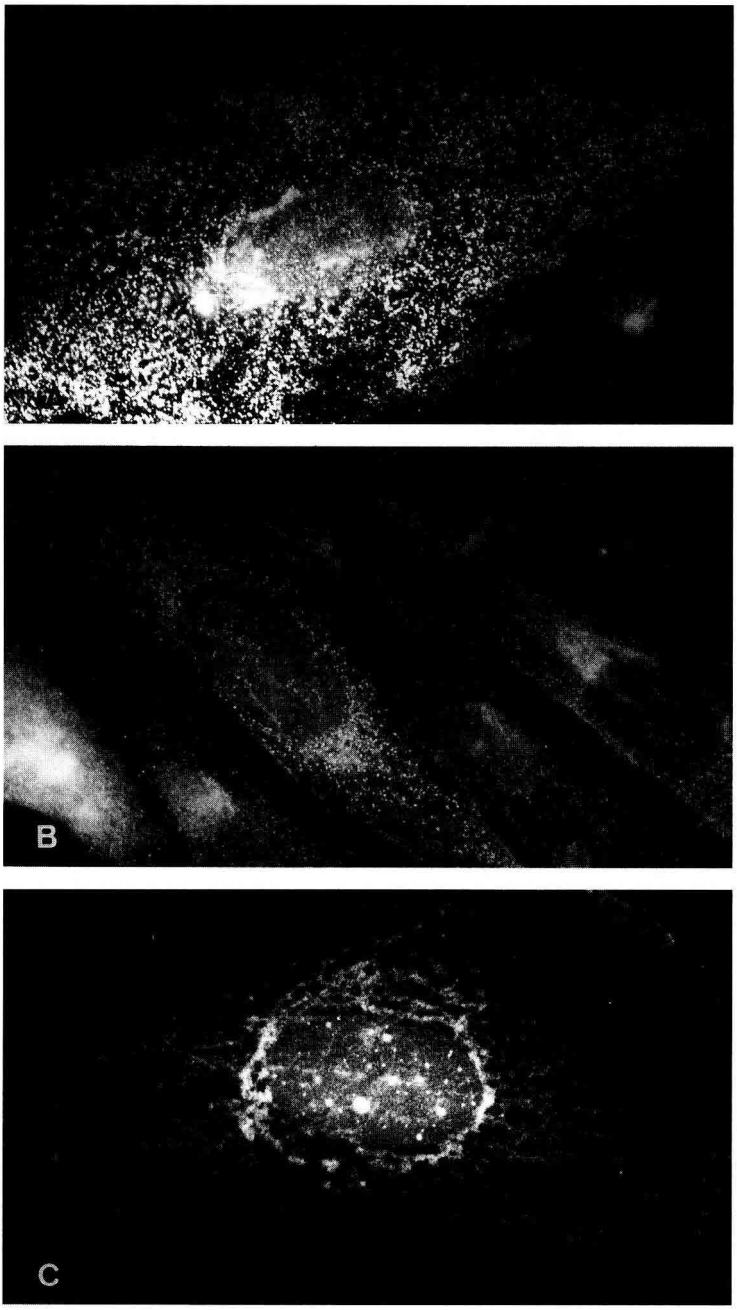

Figure 5. Granular staining pattern for basic fibroblast growth factor (bFGF) was found in the cytoplasm of proliferating rabbit (**A**) and mouse primary embryonic fibroblasts (PEF) (**B**). The granulation was clearly concentrated around the nucleus in mouse PEF treated with mitomycin C (**C**), whereas non-proliferating rabbit PEF showed no change of the staining pattern (× 450).

In mouse ES cells, $\alpha_6\beta_1$ integrin (a major LAM receptor), $\alpha_5\beta_1$ integrin (receptor for FN) and $\alpha_3\beta_1$ integrin (multi-specific receptor for LAM, FN and COL) were detected [7]. Thus, a main prerequisite for any effect of ECM proteins on ES cells is fulfilled and a different behaviour of ES cells cultured on the rabbit and mouse PEF feeders can be expected. Our following preliminary experiment confirmed this assumption. Mouse D3 ES cells [10] cultured on the rabbit PEF feeder layer (high FN, low LAM and COL IV) showed higher proliferation (detected by BrdU staining) and a lower level of differentiation (as judged on the basis of TEC-1 and CYT 18 positivity) in comparison with the ES cells cultured on the mouse PEF feeder layer (low FN, high LAM and COL IV). This result corresponds well with data about the opposite effect of FN and LAM on cell proliferation and differentiation [2, 59, 60]. As both rabbit and mouse PEF also expressed bFGF approximately at the same levels and since bFGF receptors (FGF R1, R2 and R3) were found in undifferentiated mouse ES cells [35], there is probably no differential effect in this case. Of course, the rabbit and mouse PEF probably synthesise many other proteins, which can effectively modify behaviour of cultured cells. Further studies dealing with feeder cell characterisation from the viewpoint of their protein spectrum are thus desirable to select the most suitable feeder for individual types of cultured cells.

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