Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude mice by matrigel and fibroblasts

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Summary The failure of MCF7 cells to induce the formation of tumours after sub-cutaneous inoculation into athymic nude mice can be obviated by the simultaneous injection of an extract of basement membrane proteins (matrigel). Tumour growth is promoted and the latency period is low (2 to 4 weeks). In the absence of matrigel, the simultaneous inoculation of fibroblasts and MCF7 cells also resulted in the development of tumours, but with a longer latency period (about 2 months). The tumorigenic synergy between matrigel and fibroblasts was evidenced by co-inoculating MCF7 cells MDA-MB-231 cells with fibroblasts and matrigel. This co-inoculation decreased the delay of appearance of the tumours and/or accelerated the tumour growth, depending upon the number of fibroblasts injected. Repeated injections of fibroblasts conditioned medium, at the site of inoculum of tumour cells also enhanced tumour growth, suggesting the involvement of soluble factors secreted by fibroblasts. Histologically, tumours induced by co-inoculation of tumour cells and fibroblasts contained more stromal structures including vimentin-positive cells, fibronectin and interstitial collagens. These data suggest that human tumours may be reconstituted and grown in athymic nude mice using basement membrane components and fibroblasts as inducers.

The appropriate nature of the microenvironment is one of the factors involved in the ability to transplant human tumours into athymic nude mice. Matrigel, an extract of basement membrane proteins, allows the development of tumours after transplantation of various cell types including small cell lung carcinomas (Fridman et al., 1990), human mammary cancer cells (Fridman et al., 1991), prostatic carcinoma PC3 cells and human primary prostatic carcinomas (Pretlow et al., 1991). We have previously demonstrated the rapid development of tumours in nude mice after injection of human mammary carcinoma MCF7 cells in the presence of matrigel and their responsiveness to oestrogen (Noël et al., 1992c). In the absence of this basement membrane matrix, MCF7 cells failed to induce the appearance of tumours.

Tumours are often infiltrated by cells arising from the host such as macrophages, endothelial cells, lymphocytes and fibroblasts. These cells represent an additional microenvironment element able to modulate growth and other properties of tumour cells. Co-injections of fibroblasts with human epithelial tumoral cells from various tissues have been reported to enhance tumour growth and their metastatic capacity (Picard et al., 1986; Horgan et al., 1987; Camps et al., 1990). These data suggest that several factors including tumour cell-matrix interactions (Liotta, 1984), host cell-tumour cell interactions (Picard et al., 1986; Horgan et al., 1987; Camps et al., 1990; Miller et al., 1988; Price & Zhang, 1990) may affect tumour growth and the metastatic process.

We have investigated the influence of normal human fibroblasts on human breast cancer cells (MCF7 and MDA-MB-231 cells) transplanted into nude mice in the presence or not of matrigel. This report clearly demonstrates that fibroblasts enhance the tumorigenicity of human breast cancer cells in vivo. The promoting effects of fibroblasts and matrigel are cumulative. The increased tumorigenicity observed by co-inoculating fibroblasts and tumour cells could be at least partly reproduced by medium conditioned by fibroblasts.

Materials and methods

Matrigel

Basement membrane proteins (matrigel) were prepared from dialysed urea extract of EHS (Engelbreth-Holm-Swarm)

Cells

Normal human skin fibroblasts were obtained by outgrowth from explants and used between passages 4 and 12. The human breast carcinoma cell lines, MCF7 cells (Soule et al., 1973) and MDA-MB-231 cells (Cailleau et al., 1974) were kindly provided by Dr Leclercq (Bordet Institute, Brussels, Belgium) and Dr R. Gol (University of Liège, Belgium), respectively. Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (Gibco), glutamine (292 mg ml⁻¹), sodium bicarbonate (2,1 g l⁻¹), ascorbic acid (50 μg ml⁻¹) and penicillin-streptomycin (100 U ml⁻¹).

Preparation of conditioned medium (CM)

After three washings to eliminate serum, 8 ml of serum-free medium was added to confluent monolayer of fibroblasts in 10 cm Falcon plastic dishes. The medium was collected after 24 h, centrifuged to eliminate cell debris and used immediately.

In vivo studies

Tumoral cells and/or fibroblasts were detached by trypsinisation, harvested by centrifugation, resuspended in serum-free medium and mixed with 0.25 ml of matrigel (10 mg ml⁻¹) in a total volume of 0.5 ml. Cells were injected subcutaneously (SC) into 6 to 8 week-old female athymic (nu/nu) mice (Iffa Credo). The estradiol-dependent MCF7 cells were inoculated into mice previously implanted SC with Silastic capsules (Dow Corning) containing estradiol as previously described (Robinson & Jordan, 1989; Noël et al., 1992c). In some assays, MCF7 cells were mixed with matrigel and 0.1 ml of conditioned medium (CM) of fibroblasts. CM (0.1 ml) was reinjected weekly at the site of the primary inoculum and inside the tumours after their appearance.

Injected mice were examined weekly and tumour volume was calculated as previously described (Attila & Weiss, 1966; Noël et al., 1992c). The latency period was defined as the time between injection and appearance of a 250 mm³ nodule which
will maintain a progressive growth. Results are expressed as
the mean of the tumour volumes. Tumours presenting a
volume lower than 250 mm³ (determining the latency period)
were not taken into account because of technical imprecisions
of the measurements (Noël et al., 1992c).

Each experiment was repeated at least three times (control
+ experimental variations), at three months intervals, by
using different batches of cultured MCF7 cells, fibroblasts
and distinct groups of nude mice (Ifra Credo). Each set of
animals, in each condition contained at least five to ten
individuals. Absolute values of tumour sizes in identical
groups could vary as much as 30% between each experiment,
probably due to uncontrolled variations (food intake, light-
ing, seasonal variations, environmental stress, temperature of
the unit, ...). In any case, the absolute trends of variations
between the groups in each assay remained consistant and
inter-groups variations were of the same extent. The results
presented are representative experiment with absolute values
(n = 5 to 10). Inter-individual variations of tumour size inside
each group (n = 5 to 10) were always lower than 10%.

No death occurred during the course of the experiments.
The tumours sizes were always maintained below 1,500 mm³
since such large tumours usually displayed extensive area of
central necrosis, ulcerations and subsequent infections and
death (data not shown). We therefore decided to finish the
assays when appropriate or when the tumours reached
1,250 mm³.

Histological examinations and immunohistochemistry

The tumours were excised, fixed in 10% buffered formalin,
embedded in paraffin, sectioned at 4 mm intervals and stained
with hematoxylin and eosin. The immunohistochemistry was
performed as previously described (Noël et al., 1992c). For
the characterisation of the extracellular matrix, sections
were pretreated with pepsin (1 mg ml⁻¹ in 0.01 N HCl, 10 min at
37°C). Antiserums directed against vimentin (Calbiochem),
fibronectin and types I and III collagen were raised in rabbit
(Noël et al., 1992b). The anti-Thy antibody, kindly provided
by J. Boniver (University of Liège, Belgium) was specific for
the murine fibroblasts (Esterre et al., 1992). This species
specificity was verified in culture of human and murine
fibroblasts.

For transmission electron microscopy, small pieces of
tumour tissue (1 mm³) were fixed in 2.5% glutaraldehyde and
postfixed in 0.1 M osmium tetraoxide. After dehydration into
a graded series of ethanol, samples were embedded in Epon.
Ultrathin sections were contrasted with uranyl-acetate and
lead-citrate before examination with a JEOL 100 CX II
electron microscope (60 kV).

Quantification of collagen in tumours

Tumours were excised, frozen in liquid nitrogen and
lyophilised. Their dry weight was then determined. The
amount of hydroxyproline was measured by the method of
Bergman & Loxley (1963), after hydrolysis in 6 N HCl.

Statistical analysis

Differences between the experimental conditions were
evaluated using Student's t-test (P values lower than 0.02
were considered as significant).

Results

Effect of fibroblasts on human mammary cancer cells
tumorigenicity

Athymic nude mice were inoculated subcutaneously (SC)
with estradiol-dependent MCF7 cells in various experimental
conditions. In the absence of matrigel, we did not succeed in
producing tumours in the 15 nude mice injected with MCF7
cells alone (Noël et al., 1992a), even when high number of
MCF7 cells (3 x 10⁶) were inoculated. However, co-injection
of 1 x 10⁶ fibroblasts with 0.35 or 1.5 x 10⁶ MCF7 cells
resulted in tumour development after approximately 2
months. This promoter effect of human fibroblasts was
similar for the two numbers of MCF7 cells inoculated
(Figure 1a and b; Table I).

Addition of matrigel induced a more rapid tumour take,
even when MCF7 cells were injected, without fibroblasts.
Tumours appeared during the first month of observation.
The latency period was 22 and 35 days after injection of
1.5 x 10⁶ and 0.35 x 10⁶ MCF7 cells, respectively (Table I).
Co-injection of fibroblasts with a low number of MCF7 cells
(0.35 x 10⁶ cells) in the presence of matrigel shortened the
latency period (20 vs 35 days) (Table I; Figure 1a). The
latency period observed for 1.5 x 10⁶ injected MCF7 cells,
was not modified when fibroblasts were added (Table I;
Figure 1b). However, independently of the number of MCF7
cells injected simultaneously with fibroblasts, the incidence
tumour always reached 100% and the volume of the tumours
was increased (Table I; Figure 1a and b). After 70 days
of observation, the volume reached 1,250 mm³ in the presence
of fibroblasts and only 750 mm³ in the absence of fibroblasts
(P<0.01).

The effect of fibroblasts on the tumorigenicity of an
estradiol-independent mammary cancer cell line (MDA-MB-
231) was also investigated in the presence of matrigel.
Inoculation of 1 x 10⁶ fibroblasts simultaneously with
0.35 x 10⁶ MDA cells shortened again the latency period
and increased the tumour growth (Figure 2) (P<0.02).
The injection of fibroblasts alone used as control in the presence
or the absence of matrigel did never induced the development
of tumour.
Table 1 Tumorigenicity of MCF7 cells after subcutaneous injection into athymic nude mice in the presence or absence of fibroblasts

| Tumorigenicity (n/n) | Latency period (days)* |
|----------------------|------------------------|
|                      | Without fibroblasts    | With 1 x 10^6 fibroblasts |
| Number of MCF7 cells:|                        |                           |
| in the absence of matrigel |                      |                           |
| 0.35 x 10^6          | 0/5                    | 3/5                       |
| 1.5 x 10^6           | 0/5                    | 3/5                       |
| Number of MCF7 cells:|                        |                           |
| in the presence of matrigel |                    |                           |
| 0.35 x 10^6          | 10/15                  | 15/15                     |
| 1.5 x 10^6           | 8/10                   | 10/10                     |

*Number of mice bearing tumour larger than 250 mm²/total number of injected mice. *Latency period: time between injection and appearance of 250 mm² nodule.

Dose-dependence of fibroblasts effects

When a constant number of MCF7 cells (0.3 x 10^6) was injected with different numbers of fibroblasts (from 0.3 x 10^6 to 0.9 x 10^6), in the presence of matrigel, the effect on tumour take and on tumour growth (Figure 3) was related to the number of fibroblasts. The effect was maximal at a tumoral cells to fibroblasts ratio of 1 to 2 or 3 (P<0.01).

Figure 2 Effect of co-inoculation of fibroblasts and MDA-MB-231 cells upon tumour growth, in the presence of matrigel. The MDA-MB231 cells (0.35 x 10^6) were injected into athymic nude mice alone (○) or with 1 x 10^6 fibroblasts (+), in the presence of matrigel. The tumour volume was measured as described in Material and methods. Interindividual variations of tumour sizes inside each group (n = 5) were always lower than 10%. The experiment has been repeated three times.

Effect of fibroblasts conditioned medium (CM) on MCF7 cells tumorigenicity

In an attempt to determine if the promoting effect of fibroblasts results from the production of soluble factors, MCF7 cells suspended in conditioned medium (CM) of fibroblasts were injected with matrigel. This treatment was followed by a weekly injection of CM at the primary site of inoculum. The latency period was not modified as compared to injection of tumoral cells alone (Figure 3). However, after repeated injections of CM, the growth rate and tumour size were increased (P<0.01). After 70 days of observation, the volume reached in these conditions was similar to that obtained after co-inoculation of both cell types at a 1 to 3 MCF7 cells to fibroblasts ratio (Figure 3).

Light and electron microscopy of tumours

The histology of tumours developed after injection of MCF7 cells in the presence of matrigel were studied by light and electron microscopy. Tumours appeared to be well circumscribed. Cells were organised into nodules of malignant cells with very few stromal cells (Figure 4a). Numerous mitotic figures were regularly observed. Despite an extensive vascularisation, central necrosis developed. In other areas of the tumour, cells lined up between stromal elements (Figure 4b). The tumours developed after the simultaneous injection of fibroblasts and MCF7 cells in the presence of matrigel were characterised by the regular presence of more abundant stromal structures (Figure 4c).

By electron microscopy, tumour cells presented features of malignant MCF7 cells (high nuclear cytoplasmic ratio, filaments arranged in bundles, numerous mitochondria) (Figure 5a,b). Infiltration of fibroblasts characterised by their abundant rough endoplasmic reticulum was observed in tumours induced by injection of MCF7 cells alone (Figure 5a,b) or in the presence of fibroblasts (Figure 5c,d). In some areas, cells were separated by a granular material resembling matrigel (Kleinman et al., 1986; Noél et al., 1991). Fibrillar striated materials was found at the vicinity of fibroblasts (Figure 5d). Cells were connected to desmosomes-like junctions, interdigitating cytoplasmic projections or the membrane of neighbouring cells were in juxtaposition (Figure 5d). Vascularisation was evidenced in tumours obtained in both conditions (Figure 5e).

Characterisation of stromal structures

Since tumours induced by co-inoculation of fibroblasts and tumour cells displayed more stromal structures, the extracellular matrix deposition and stromal cells were characterised by immunohistochemistry. Interstitial collagen types I and III and fibronectin were evidenced in these structures surrounding epithelial tumour cells (Figure 6a). The content of collagen was measured as hydroxyproline present in tumour lysates. The tumours were excised 25 and 40 days after inoculation of MCF7 cells in the presence or absence of fibroblasts. The
collagen content of tumours developed after co-inoculation of both cell types was double that of tumours induced by injection of MCF7 cells alone (92 ± 20 μg collagen mg⁻¹ dry weight vs 50 ± 10 μg collagen mg⁻¹ dry weight; P < 0.005). Fibroblasts stained positively for vimentin. Surprisingly, the fibroblasts as shown by using anti-Thy 1 antibodies revealed to be exclusively of murine origin (Figure 6b). This antibody is specific for murine fibroblasts and not for human fibroblasts, as verified on our fibroblasts in culture (data not shown). All stromal cells stained positively with this antibody.

Similar histological features were observed after repeated injections of CM of fibroblasts, at the site of inoculation of MCF7 cells with matrigel (Figure 4d).

Discussion

In this study, we demonstrate that the addition of fibroblasts to MCF7 cells which are not tumorigenic by themselves allows the take and growth of tumours. These data confirm the helper effect of normal fibroblasts as observed for different types of tumour cells of animal and human origin derived from breast (Horgan et al., 1987; Camps et al., 1990), rhabdomyosarcoma (Picard et al., 1986), melanoma (Tanaka et al., 1988), prostate and bladder (Camps et al., 1990). However, tumours induced by co-injection of MCF7 cells and fibroblasts appeared only after 2 months, in a rather small proportion of injected animals and grew slowly (Table I and Figure 1). More recently, matrigel has been shown to accelerate tumour growth in athymic nude mice (Fridman et al., 1990; 1991; Pretlow et al., 1991). We observed similar results since tumours developed 2 to 4 weeks after injection of MCF7 cells in the presence of matrigel (Noël et al., 1992c). The exact mechanisms operating in the enhancement of tumorigenicity by matrigel are not clear and have been previously discussed (Fridman et al., 1990; 1991; Noël et al., 1992c). The effect of matrigel on the human cells tumorigenicity was partially abolished by the addition of a
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Figure 5  Electron micrographs of tumours obtained after injections of MCF7 cells in the presence of matrigel into athymic nude mice. a,b, Tumour obtained after injection of MCF7 cells (M) alone. Rare presence of fibroblasts (F) was observed (bar = 1 μm). c–d, Tumour obtained after injection of MCF7 cells (M) and fibroblasts. Fibroblasts (F) were surrounded by striated fibrillar material (c), (bar = 1 μm). Cells were connected by desmosome (arrow) or interdigitating cytoplasmic projections (d) (bar = 0.5 μm). e, Capillary surrounded by extracellular matrix and tumoral MCF7 cells. (bar = 100 μm).

synthetic peptide from the B1 chain of laminin (Fridman et al., 1990) suggesting that it could be at least partly ascribed to specific cell-matrix interactions promoted by laminin (Noël et al., 1988, 1993).

When fibroblasts were inoculated simultaneously with a low number of MCF7 cells in the presence of matrigel, the latency period was reduced and the tumour growth was increased. These effects are correlated with the ratio of tumoral cells to fibroblasts. In these conditions, all mice developed tumours. Similar results were obtained with an other breast cancer cell line (MDA-MB-231) and are not restricted to the MCF7 cell line. The factors supplied by fibroblasts and matrigel are cumulative as shown by the results obtained with a low number of tumoral cells. Furthermore, injections of higher amounts of matrigel or repeated injections of matrigel were unable to mimic the effect of fibroblasts (data not shown). These results suggest that fibroblasts and matrigel enhance tumour growth by distinct mechanisms. Identical tumour sizes were obtained when 0.3 × 10⁶ or 3 × 10⁶ MCF7 cells were inoculated indicating that the tumour growth was not proportional to the number of MCF7 cells injected. The identical latency periods observed when 1.5 × 10⁶ MCF7 cells were injected in the presence of matrigel, with or without fibroblasts suggest that the latency period is minimal or that local host factors limit the rate of tumour take.

Histological observations demonstrated the presence of fibroblasts and stromal-like structures inside of the tumour islets. This stroma contained fibronectin, collagen types I and III as evidenced by immunohistochemistry. The content of total collagen was twice as high in tumours induced by co-inoculation of tumour cells and fibroblasts. This could be ascribed at least partly to a modulation of collagen production by fibroblasts in response to MCF7 cells. We have indeed demonstrated the capacity of these mammary tumour cells to secrete soluble factors which enhance collagen synthesis by fibroblasts in vitro (Noël et al., 1992a,b).

The murine origin of fibroblasts surrounding epithelial
mediated fibroblasts indicate histological specificities following injections of anti-Thy polyclonal surrounding matrigel in nude mice. Various cell or motility recruitment of the tumours was observed by Yee et al., 1988) by skin fibroblasts might be responsible for this paracrine effect (Van Roozendaal et al., 1992). Furthermore, the matrix, known to bind several cytokines via the heparan sulfate proteoglycan (Noël et al., 1992a; Taub et al., 1990; Vukicevic et al., 1992) could act as a 'reservoir' accumulating these intercellular messengers.

The maximal stimulation is obtained when the tumours are in contact with fibroblasts. It is likely that maximal stimulation of tumorigenicity requires the continuous production of factors by fibroblasts and cell-cell contacts. The importance of these interactions between tumour cells and fibroblasts have been suggested by several studies (Gartner et al., 1992; Tanaka et al., 1988; Coucke et al., 1992). The lung colonising potential of a low metastatic clone of melanoma cells was indeed increased when cells were cocultured in vitro with fibroblasts. The CM of cocultured melanoma cells and fibroblasts presented similar potential whereas medium from monoculture of fibroblasts showed only a low activity (Tanaka et al., 1988). We have previously demonstrated that matrigel promotes not only cell-matrix interactions but also cell-cell interactions in MCF7 cell culture (Noël et al., 1988). The same gel also operated in tumour cell-fibroblasts interactions since in coculture in vitro, MCF7 cells organised into clusters attached on top of fibroblasts aggregates (Noël et al., 1993). These interactions are modulated by both the soluble and insoluble forms of laminin and fibronectin. In addition, when cultured on matrigel, fibroblasts have been shown to deposit extracellular material, resulting in a progressively more fibrillar pattern of the matrix gel (Emonard et al., 1987). Fibroblasts are also known to actively organise the network of interstitial type I and III fibrils (Bell et al., 1979). Such rearrangements of the tissue architecture might also modulate tumour growth.

In conclusion, our results emphasise the importance of tumour-host interactions and mainly basement membrane proteins and fibroblasts or their synthetic products on cells tumorigenicity. Our work also suggests that human tumours may be reconstituted and grown in athymic nude mice using stromal and basement membrane components as inducers. This model may be helpful in the study of factors mediating cellular interactions during neoplastic progression and for testing anticancer agents.

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Figure 6 Immunoperoxidase stainings of tumours obtained after injections of MCF7 cells and fibroblasts, in the presence of matrigel into athymic nude mice. a, Immunostaining with a polyclonal antibody localises the collagen type I in the stroma surrounding tumour cells. b, Immunostaining of fibroblasts with anti-Thy 1 antibody raised against human fibroblasts antigen. (bar = 1 μm)

tumour nests was determined using an antibody staining specifically murine fibroblasts. Furthermore, the identical histological features of tumours induced by addition of fibroblasts or by injections of medium conditioned by fibroblasts indicate recruitment of murine fibroblasts rather than a persistence of the injected human fibroblasts. In this regard, fibroblasts have been shown to secrete several factors affecting cell motility (for review, Stocker & Gherardi, 1991).

Various mechanisms may be involved in the fibroblasts-mediated increase of tumorigenicity. Camps et al. (1990) reported that lethally irradiated fibroblasts retain at least part of their potential to accelerate tumour growth, suggesting the involvement of the bio matrix surrounding the cells. This effect could be ascribed to specific properties of its components or to its capacity to bind growth factors (Nathan & Sporn, 1991). For example, several forms of fibroblasts growth factors (FGF) are deposited in the extracellular matrix and function when bound to proteoglycans of the extracellular matrix (Nathan & Sporn, 1991; Bashkin et al., 1989). According to previous observations (Picard et al., 1986) and to our results, fibroblasts conditioned medium (CM) mixed with tumour cells also stimulates tumour growth (Figure 3). The failure of CM to shorten the latency period could be ascribed to subeustaneous dilution of soluble factors before tumour take (Gartner et al., 1992). Nevertheless, these data suggest the role of soluble growth factors secreted by fibroblasts. The production of IGF-I and IGF-II (Clemmons, 1984; Yee et al., 1988) by skin fibroblasts might be responsible for this paracrine effect (Van Roozendaal et al., 1992). Furthermore, the matrix, known to bind several cytokines via the heparan sulfate proteoglycan (Noël et al., 1992a; Taub et al., 1990; Vukicevic et al., 1992) could act as a 'reservoir' accumulating these intercellular messengers.
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