Fibroblast-dependent Tumorigenicity of Cells in Nude Mice: Implication for Implantation of Metastases

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ABSTRACT

The inadequate nature of the microenvironment is one of several factors considered in the failure of tumor engraftment in athymic mice; in the present work, we have tried to more adequately reconstitute it by injecting tumor cells together with fibroblasts.

We have demonstrated that the s.c. co-inoculation of fibroblasts with different kinds of tumor cells of animal origin [rat rhabdomyosarcoma (RMS) 9-4/0, rat hepatocarcinoma FAO] or human origin (colonic adenocarcinoma HT29, Ewing's sarcoma pleural metastasis EW-S1) is necessary for tumor take and growth when the number of tumor cells alone is below the tumorigenic dose. We have shown that the s.c. co-inoculation of 10⁶ fibroblasts and 10³ RMS 9-4/0 tumor cells induced a tumor in all the recipient mice, while 10² tumor cells alone never gave any tumor. With a tumorigenic number of RMS 9-4/0 tumor cells (10⁴), addition of 10⁶ fibroblasts decreased the delay between cell injection and tumor appearance, thereby increasing tumor take and growth rate. These results were observed not only in nude animals (mice and rats) used as recipient animals but also in normal WAG rats receiving the syngeneic RMS 9-4/0 tumor cells, and they were independent of the nature or origin of the different fibroblasts.

This helper effect has also been observed in the normal WAG rats. I.v. injection of tumor cells from a poorly metastatic 9-4/8 subline, derived from the RMS 9-4/0 line and mixed with 10⁴ fibroblasts, gave a high number of lung colonies. Addition of 10⁶ irradiated 9-4/8 tumor cells instead of fibroblasts did not increase the lung colonizing potential.

Fibroblast-conditioned medium mixed with tumor cells instead of fibroblasts also enhanced tumor take and size but to a lesser extent than did the fibroblasts themselves.

Only endothelial cells cultured from porcine aorta had a similar helper effect among the cells tested.

It is argued in the discussion that the proliferating state of cultivated fibroblasts is a determinant factor concerning upon them the ability to promote tumor cell growth, while fibroblasts very numerous at the implantation site but quiescent might not be efficient in cooperation. Changes in fibroblast morphology and physiology may be necessary in order for tumor cells to express their tumorigenicity.

INTRODUCTION

Although the nude mouse is an unnatural host for xenogenic neoplasms, it provides a very useful model for demonstrating the tumorigenic capacity of cells (1, 2) and allows for the study of tumor cell properties (3) and host factors which influence it (4, 5). We shall here consider the microenvironment resulting from diverse factors which are available to interact with tumor cells (6, 7), both locally such as the stroma and surrounding genes in the case of viral transformed cells. This failure of such as, for example, their ability to grow in agar, the absence of cell contact inhibition, and the acquisition of growth factor independence for cell proliferation or in the presence of viral genes in the case of viral transformed cells. This failure of tumor take has long been attributed to spontaneous, non-immunological rejection by these T-cell deficient animals. Natural killer cell responses have been supposed to play the main role in the tumor rejection (5, 12, 13). Indeed, several investigations have shown that tumor cell injection into very young nude animals deficient in natural killer lymphocytes or into adult nude animals treated by natural killer inhibitors such as diethylstilbestrol or anti asialo-GM1 (14) leads to a higher incidence of tumor take. However, even in such treated animals, some tumor cell lines or in vitro-transformed cells do not always form a tumor after injection, as we observed in our laboratory. The source of this failure might be sought in the lack of tumorigenicity of the cells or in the inadequate nature of the environment (15).

The unfavorable nature of a microenvironment which is unsuitable for tumor cell grafts has also been suggested, thus requiring that the expression of tumorigenicity (which incidentally is not always associated with transformation) may depend on returning the cells to their natural environment. Thus colon cancer cells should be implanted in the gut (4), rhabdomyosarcoma cells in the muscle, melanoma cells in the skin, and so on.

The importance of the number of injected tumor cells in the tumor take is well known. For each tumor cell line, there is a relatively stable tumorigenic threshold which differs from one tumor line to another. This question of a limiting number of tumor cells necessary for inducing a tumor take is crucial, especially in the case of blood borne potentially metastatic tumor cells. When tumor cells have to implant themselves in a metastatic site (16, 17), the small number of spontaneously circulating cells could be a very limiting factor. The environment, however, can be a decisive factor in the establishment of metastasis (18), as well as in the tumor take after s.c. injection of a small number of tumor cells.

The aim of this work was to reconstitute a better adapted microenvironment by injecting tumor cells mixed with fibroblasts. The tumor cells used originated in a rat rhabdomyosarcoma (19), a rat hepatocarcinoma (20), a human adenocarcinoma, or a human Ewing's sarcoma (21). In this paper we demonstrate that addition of fibroblasts to tumor cells was necessary for tumor take, when the number of tumor cells alone was insufficient to produce tumors. We hypothesized that interactions between tumor cells and fibroblasts may be necessary in order for tumor-derived cells to initiate a tumor graft in the host.

MATERIALS AND METHODS

Cell Culture. Rhabdomyosarcoma 9-4/0 cells were isolated by trypan blue exclusion. In primary culture the cells were grown in Dulbecco's modified Eagle's medium (H21, from Grand Island Biological Co., Grand Island, NY) containing 1% glutamine, 10% heat-inactivated fetal calf serum and penicillin (100 U/ml), streptomycin (100 µg/ml). Confluent cultures were maintained on a monolayer by subpassages. The 9-4/8 line, a weakly metastatic tumor cell line, was derived by cloning from the RMS² 9-4/0 line, sharing the same culture conditions.

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2 The abbreviation used is: RMS, rhabdomyosarcoma.
HT 29 cells were isolated from a human colon adenocarcinoma (FOJ, FOGH; Memorial Sloan-Kettering Center for Cancer Research, 1964). A continuous cell line was obtained and maintained in culture sharing the same culture conditions as described above.

EW-S1 cells were isolated from an Ewing's sarcoma pleural metastasis (21). A continuous cell line was obtained and maintained in culture, sharing the same culture conditions.

FAO cells are a well differentiated subclone derived from the rat hepatoma H 35 (20) and kindly provided by J. Deschatrette (CNRS, Gif-sur-Yvette, France). A continuous cell line was obtained and maintained in culture with the same conditions.

Fibroblasts and other non-tumoral cells were obtained as follows. Rat fibroblasts of WAG, Fisher, and nude rat origin and mouse fibroblasts from the nude BALB/c strain were prepared from 14-day-old embryos after head and liver resection and trypsin treatment. They were cultured in the above-described medium maintained in monolayer by successive passages and used before the 8th passage in culture.

Human embryo fibroblasts, provided through the courtesy of A. Maceira-Coelho (22), were used in the same culture conditions. In the experiments indicated in the text, fibroblasts were submitted to various treatments. A fibroblastic cell suspension was either treated at 56°C for 30 min before mixing with the tumor cells or fixed with a 2% glutaraldehyde solution for 30 min and washed twice in phosphate-buffered saline before the co-injection. Another aliquot of fibroblasts was irradiated at 10,000 rads.

Macrophages were extracted from WAG rat bronchoalveolar fluid by extensive washing in 0.2% EDTA in phosphate-buffered saline. After centrifugation, the macrophages were suspended in the same medium and used for the experiment without being subcultured. Endothelial cells from porcine aorta were extracted using the technique of Joffe (23), modified by Pearson (24). They shared the same culture conditions as described for tumor cells and were used at 4th passage (kindly provided by S. Korach, I.G.R., Villejuif, France).

Preparation of Conditioned Medium. Confluent fibroblast monolayers in 10-cm Falcon plastic dishes were renewed with serum-free media. These media were collected 24 h later and filtered through a 0.22-μm filter. A 0.1-ml sample was mixed (v/v) with suspended tumor cells from 20 to 60 min before injection and kept on ice until injection; 0.1 ml of the conditioned medium was reinjected three times at the site of the primary inoculum at 2-day intervals, compared to 0.1 ml of non-conditioned culture medium for the control group.

Ten- to 12-week-old male and female nude mice with a BALB/c × Swiss genetic background were used as graft recipients and received an ad libitum complete diet for laboratory animals from Piètrement Co. (77, Provins, France) and 0.22 nM Millipore filtered water. Ten- to 12-week-old male or female inbred WAG and nude rats were used. They were from the house-breeding of the Institut de Recherches Scientifiques sur le Cancer at Villejuif, France and were maintained in specific pathogen free conditions.

Injection Procedure. Tumor cells and fibroblasts harvested after trypsin treatment of subconfluent monolayers were washed once in Dulbecco’s modified Eagle’s serum-free medium and counted. The suitable number of tumor cells was contained in 0.05 ml or with 0.05 ml of medium. Then, the mice were given s.c. or i.v. of 0.1 ml of the conditioned medium was reinjected three times at the site of the primary inoculum at 2-day intervals, compared to 0.1 ml of non-conditioned culture medium for the control group.

Tumor Growth Measurement. Tumors were measured twice per week. The mean of the two major diameters was recorded as the tumor size. The delay between injection and tumor appearance was carefully noted. If mice died, then the mean (tumor size) diameters were measured with the animals which were still alive. Statistical analysis was performed according to Student’s t-test.

RESULTS

Kinetics of Tumor Growth in Nude Mice after s.c. Injection with 9-4/0. Inocula established as growing tumors showed a characteristic period of latency of 15 days.

This period was followed by exponential growth which continued up to a maximal size (1), after which the tumor became necrotic and ulcerated and remained at a plateau.

Between cell inocula of 10² and 10⁴ cells, there was a correlation between the number of injected cells and tumor take (Fig. 1); above 5 x 10⁴ all mice developed tumors, and above 10⁴ 80% of mice died. For a great number of grafted mice, the established curve showed that there was no tumor take for a number of injected cells below 10⁵ (10³ or 5-10⁴). For the usual dose of 10⁵ cells, we obtained a tumor take of 36%. The injection of 5.10⁵ cells induced tumor growth in 60% of the mice. The calculated inoculum size which induced tumor take in 50% of the mice was approximatively 2500 cells (see Fig. 1).

Effect of s.c. Co-inoculation of Fibroblasts on Appearance and Growth of Tumor in Nude Mice. The injection of rat WAG fibroblasts together with the RMS 9-4/0 tumor cells resulted in tumors in all treated mice, even when as few as 10⁴ tumor cells were injected, greatly decreasing the tumorigenic threshold (Fig. 2; Table 1). The period of latency between the injection of 10⁴ tumor cells and tumor appearance was considerably shortened, to 5 days, by co-injection of 10⁴ fibroblasts. No differences were seen upon histological examination between tumors induced by injection of tumor cells alone and co-inoculation with fibroblasts. The injection of fibroblasts alone, of course, never allowed any tumor outcome.

Effect of Co-inoculation of RMS 9-4/0 Tumor Cells with Medium Conditioned by Fibroblasts. The co-injection of fibroblast-conditioned medium with tumor cells also resulted in increased tumor take frequency; 60% (12 of 20) of mice had tumors between the 20th and 30th day following co-injection of 10⁴ tumor cells and conditioned medium, while only 12% (2 of 16) had tumors after injection of 10⁴ tumor cells alone. There was also an increase in growth rate and tumor size compared to the injection of tumor cells alone, but this remained lower than that observed after co-inoculation with fibroblasts (Fig. 3).

Effect of Co-inoculation with Irradiated Fibroblasts, Irradiated RMS 9-4/0 Tumor Cells, or Killed Fibroblasts. Irradiated WAG
FIBROBLAST DEPENDENT TUMORIGENICITY

Table 1 Effect of s.c. co-inoculation of fibroblasts and tumor cells upon tumor growth

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of tumor cells</th>
<th>No. of fibroblasts</th>
<th>No. of mice bearing a tumor/total no. of mice</th>
<th>Tumor mean size (mm)</th>
</tr>
</thead>
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<tr>
<td>RMS 9-4/0</td>
<td>10^6</td>
<td>0</td>
<td>3/6</td>
<td>5.7 ± 0.8*</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>10^6</td>
<td>10/10</td>
<td>15.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>0</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>10^6</td>
<td>6/6</td>
<td>11.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>0</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>10^6</td>
<td>8/8</td>
<td>10.0 ± 1.8</td>
</tr>
<tr>
<td>HT 29</td>
<td>10^6</td>
<td>0</td>
<td>6/10</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>10^6</td>
<td>10/10</td>
<td>9.8 ± 0.3</td>
</tr>
<tr>
<td>EW-Sn</td>
<td>10^6</td>
<td>0</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>10^6</td>
<td>5/10</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td>FAO</td>
<td>10^6</td>
<td>0</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>10^6</td>
<td>8/10</td>
<td>7.1 ± 1.4</td>
</tr>
</tbody>
</table>

* Mean of two diameters on the 30th day ± SD.

Fig. 3. Comparative effect of co-inoculation of tumor cells with fibroblasts or conditioned medium. Nude mice were given inoculations s.c. of 10^6 RMS 9-4/0 cells alone (curve 1), mixed with 10^6 Wistar AG rat embryo fibroblasts (curve 3), or mixed with conditioned medium of the same origin (curve 2). Bars, SD.

Fibroblasts were co-inoculated with the RMS 9-4/0 cells under the same conditions. The helper effect of irradiated fibroblasts on tumor growth was observed. At the 30th day after the injection, the tumor size was 11 ± 2 (SD) mm for the group of mice co-injected with tumor cells and fibroblasts; the size was about the same (10.25 ± 2 mm) for the group of mice co-injected with tumor cells and irradiated fibroblasts. At the opposite the coinjection of tumor cells with irradiated tumor cells did not give any helper effect; the size remained smaller (4.9 ± 2.2 mm). The size for the control group injected with tumor cells alone was 6.8 ± 1.8 mm (Fig. 4). Likewise the destructive treatment of fibroblasts by heating or by fixation by glutaraldehyde suppressed their helper effect.

Effect of Co-inoculation of RMS 9-4/0 Tumor Cells with Fibroblasts of Different Origins. Co-inoculation of fibroblasts other than rat WAG was performed. Several kinds of cells were injected (Table 2) with RMS 9-4/0 cells, and each type gave rise to an increased frequency of tumor take and to an increased tumor size measured 30 days after inoculation. All different fibroblasts from BALB/c nude mouse syngeneic with nude recipients had the same effect, even those of human origin.

Effect of Co-inoculation of Fibroblasts and RMS 9-4/9 Tumor Cells in the Rat. The same tumor cell number (10^6 cells) was required to obtain 100% tumor take in WAG rats and in nude mice. The co-injection of 10^6 WAG fibroblasts with 10^6 RMS 9-4/0 tumor cells was followed by tumor growth in 9 of 10 rats, while in this experiment, 10^6 9-4/0 tumor cells alone induced tumors in only 3 rats out of 8. Allogeneic non-inbred nude rats were also used as recipients. These rats did not develop tumors when given injections of less than 10^6 RMS 9-4/0 tumor cells. When 10^6 fibroblasts were added to 10^6 tumor cells, 3 rats out of 8 developed growing tumors (Table 3).

Effect on Lung Colonization of i.v. Co-inoculation of Weakly Metastatic Closed Cells (9-4/8) with Fibroblasts. I.v. injection of 10^5 RMS 9-4/8 subline cells was followed by a very limited colonization of the lungs. The median of tumor nodules counted at their surface was 2. After addition of fibroblasts to tumor cells at the 10:1 ratio, the median of tumor lung nodules increased to 204. As a control, we added 10,000 rad-irradiated 9-4/8 subline cells to the same cells, non-irradiated, at a 10:1 ratio; the median of tumor lung nodules was 14 (Table 4). The...
cells and fibroblasts, 5 mice out of 6 developed growing tumors. After treatment with the fibroblast mixture (11.3 ± 0.3), it was observed that the tumor size at day 30 remained smaller (9 ± 0.9) than that observed in the control group of mice receiving 10^6 tumor cells alone. Though the tumor incidence and an increase in the tumor growth rate, as was the case with fibroblasts. Thirteen days after the injection, 2 mice out of 6 given injections of 5 x 10^3 RMS 9-4/0 cells bore tumors. While all 8 mice treated with 5 x 10^4 RMS 9-4/0 cells mixed with 10^6 endothelial cells bore tumors. Though the tumor size at day 30 remained smaller (9 ± 0.9) than that observed after treatment with the fibroblast mixture (11.3 ± 0.3), it was higher than that obtained after tumor cell injection alone (6.6 ± 1).

**DISCUSSION**

The main conclusion is that the addition of fibroblasts to a small number of tumor cells which in themselves are unable to induce a tumor allows the take and growth of tumors. This helper effect has been demonstrated for different kinds of tumors of animal or human origin. With a large number of tumor cells, fibroblasts considerably shorten the delay (5 days instead of 15) between the injection and appearance of tumor, they increase the tumor-take frequency, and they also accelerate the growth rate. With a subtumorogenic dose (10^5) of RMS 9-4/0 tumor cells, the addition of 10^6 fibroblasts increased the tumor-take frequency from 0 to 100%. With 10^6 EW-Si cells mixed with 10^6 fibroblasts, the tumor-take frequency increased from 0 to 50% and allowed a graft which was not obtained without fibroblast addition. These data provide evidence that fibroblasts could be a useful tool to increase the tumor graft in the nude animals. Anatomical-pathological studies showed that there were no histological differences between tumors obtained after injection of a high number of tumor cells alone in the case of the rhabdomyosarcoma or tumors mixed with fibroblasts; the tumor induced by EW-Si cells and fibroblasts was diagnosed as a Ewing's sarcoma.

For RMS 9-4/0 cells, experiments showed that the optimal helper effect was obtained at a 1000:1 ratio of fibroblasts to tumor cells and was null for a ratio of 1:1. The growth of EW-Si tumors was obtained by the addition of fibroblasts in a 1:1 ratio, with an initial tumor cell number of 10^6. It is likely that the fibroblast number necessary to obtain the tumor take differs from one tumor to another. If fibroblasts allowed a tumor take with the injection of only 10^2 RMS 9-4/0 tumor cells, they never were able to produce it with a lower tumor cell number, whereas most tumors are supposed to start from a single cell. So if fibroblasts play an important role, they do not afford all of the necessary conditions for tumor take. Lynette-Wilson (9), who described this phenomenon with melanoma, observed differences in male and female hosts, probably related to the hormonal status, which we never observed with our different tumor lines (RMS 9-4/0, HT 29, FAO, EW-S1).

The same helper effect was observed also not only in the nude rat but in the normal WAG rat, leading to the conclusion that this effect is not limited only to the nude animals (rat or mice) but is a general one.

The origin and nature of the fibroblasts did not intervene. Different fibroblasts were used, including allogenic and xenogenic fibroblasts of mouse, rat, and human origin, from adults and from embryos, and all had the same effect, not surprisingly if it is a "feeder" one; more surprising is the fact that the fibroblasts were ineffective when killed by fixation with glutaraldehyde or heating, although they remained effective when irradiated. This suggests that a fibroblast mediated effect is independent of the ability of fibroblasts to divide but depends on their protein synthesis capacity.

After i.v. injection, we observed that co-injection of fibroblasts with poorly metastasizing tumor cells (9-4/8) strongly increased lung colonization, while irradiated tumor cells co-injected with viable tumor cells in the same conditions increased it very little. Fidler (18) had demonstrated that mice given injections i.v. of viable B16 tumor cells together with dead B16 or viable syngeneic embryo cells had a statistically significant higher number of pulmonary tumors as compared to mice given injections of viable B16 cells alone. From this, he concluded that the
mechanism responsible for the phenomenon might be linked to the induction of pulmonary vasospasm, the arrest of emboli, bringing about an increased entrapment of tumor cells in the lung capillaries.

Later, Hart et al. (25) demonstrated that viable tumor cells administered with non-tumorigenic irradiated tumor cells increased the lung nodules and concluded that it might be due to the overlying of specific and non-specific immune host surveillance.

In our work, the fact that there is a very strong difference when viable tumor cells are co-injected i.v. with fibroblasts (media of tumor lung nodules, 204) or irradiated tumor cells (median of tumor lung nodules, 14) would permit us to conclude that if the cells can act as a buffer and protect tumor cells, fibroblasts have an additive or synergistic effect which might be due to one or several diffusible factors (26). Indeed, a fibroblast-conditioned medium mixed with tumor cells instead of fibroblasts enhanced tumor take and increased tumor size but to a lesser degree than fibroblasts themselves. Only 12% (2 of 16) of mice had a tumor after injection of 10⁴ tumor cells alone, while 60% (12 of 20) had a tumor after co-injection of 10⁴ tumor cells and conditioned medium. This fibroblast conditioned medium could induce or contribute to the angiogenesis which may be necessary to tumor establishment, as supported by the work of Folkman et al. (27). We have observed no differences when the conditioned medium was mixed with the tumor cells just before the injection, compared to the effect of subsequent injections in the implantation site.

Other mechanisms could also be involved. Iozzo demonstrated (28) that when serum-free medium conditioned by human colon carcinoma cells was tested on quiescent fibroblasts, there was an increase in the biosynthesis of proteoglycans. Biswas (29) demonstrated that co-cultures of human fibroblasts and human tumor cells contained increased collagenase activity compared to cultures of the individual cell types. These two factors could possibly play a role in blood-borne tumor cell implantation.

Among the other cells tested as helper cells, macrophages injected s.c. with tumor cells did not give any tumor take. Fidler (30) and later Liotta and Cattozzi (31) had already suggested that i.v. injected, circulating, activated macrophages could play an inhibitory role in the hematogenous release and survival of cells from established tumors. On the other hand, endothelial cells also efficiently help in tumor take.

In conclusion, this work provided evidence that the tumorigenicity of cells, which is measured by their capacity to form growing tumors in vivo at the primary or secondary site, is related to interactions between these cells and the host. These interactions are a determining factor, and their complexity necessitates further investigations.

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