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Papers

Effects of Extracellular Matrix on the Response of Endothelial Cells to Radiation *in vitro*

Zvi Fuks, Israel Vlodavsky, Michael Andreeff, Maureen McLoughlin and Adriana Haimovitz-Friedman

The study demonstrates that the sensitivity of endothelial cells to irradiation *in vitro* is significantly affected by the microenvironmental conditions under which the experiment is carried out. When irradiated plateau phase bovine aortic endothelial cells were assayed for colony formation on top of uncoated plastic of standard culture dishes, the dose survival curves showed Do values of [mean (S.D.)] 107 (6) cGy and Dq of 63 (28) cGy (calculated according to the single-hit multitarget model). When assayed in dishes precoated with the autologous natural basement membrane-like extracellular matrix (BAEC/ECM) the curves showed a similar Do [106 (2) cGy], but the Dq was 194 (8) cGy ($P < 0.05$), indicating that components of natural ECM confer in endothelial cells an improved capacity to repair radiation lesions and to restore the clonogenic capacity. However, when the natural but biologically unrelated HR9-bFGF/ECM was used, a decreased repair capacity was noted with Dq of 156 (30) cGy ($P < 0.05$ compared with BAEC/ECM). The data demonstrate the high specificity of the repair function to interactions with autologous matrix components, and emphasise the need to select relevant experimental conditions when parameters of the radiation response *in vitro* are used to predict the response *in vivo*.
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INTRODUCTION

RECENT HISTOPATHOLOGICAL and ultrastructural studies showed that damage to the endothelium of the microvasculature is one of the predominant lesions observed during the early phases of radiation injury to slowly proliferating normal tissues, while atrophy and fibrosis characterise the late radiation damage syndrome [1–4]. Whereas the exact role of vascular damage in the pathogenesis of chronic radiation damage remains controversial [5, 6], there is evidence that microvascular degeneration and reduction in vascular density precede the destruction of other tissue elements and organ dysfunction in chronic radiation damage to the skin [7], heart [8], lung [9], liver [10], and the central nervous system [11]. Ultrastructural studies demonstrated that endothelial cells are the most sensitive targets for radiation in the vessel wall [1–4], and a spectrum of changes, ranging from early reversible endothelial cell edema and vesicle formation to advanced degeneration and cell death, have been described and correlated with time and dose parameters [1–4, 6–11]. *In vitro* studies on the sensitivity of endothelial cells to radiation have disclosed a great deal of variability. Clonogenic dose survival curves of endothelial cells obtained from various origins showed Do values ranging from 101 to 165 cGy and Dq values between 64 and 230 cGy [12–17]. Some studies also demonstrated variability in endothelial cell capacity for sublethal radiation damage repair (SLDR), with split dose to single dose ratios of 1.5–2.2, and in the capacity for potentially lethal damage repair (PLDR), with delayed plating to immediate plating survival ratios of 1.2–3.9[13, 15, 16].

The colony formation assays used to generate *in vitro* radiation dose survival curves have usually been carried out by culturing irradiated endothelial cells on top of artificial surfaces, such as the plastic surfaces of regular culture dishes [12, 14], or gelatin coated dishes [13, 15, 16]. *In vivo*, however, endothelial cells rest upon subendothelial basement membranes, that serve not only as anatomical support structures for cell anchorage and cytoskeletal orientation, but also provide and regulate physiological stimuli for a variety of normal cellular functions, cell growth and differentiation [18–22]. Tissue culture dishes coated with natural basement membrane-like extracellular matrix (ECM) have recently become available, produced by cultures of endothelial cells. When bovine aortic endothelial cells (BAEC) are cultured *in vitro* they synthesise and secrete on top of the plastic surface of the culture dish a membranous layer of ECM (the BAEC/ECM) which closely resembles in composition and supramolecular structure the vascular subendothelial basement membrane *in vivo* [18]. This matrix remains intact and firmly attached to the culture dish even following solubilisation of the endothelial cell layer with non-ionic detergents [18]. It contains heparan sulphate-bound basic fibroblast growth factor (bFGF) and exhibits normal biological activities in terms of support of endothelial cell proliferation and differentiation [19, 23]. Culture dishes precoated with BAEC/ECM thus provide *in vitro* conditions that closely resemble the normal endothelial cell microenvironment *in vivo*, but whether their use in colony formation assays would affect the shape and parameters of radiation dose-survival curves of endothelial cells is unknown.

Correspondence to Z. Fuks.
Z. Fuks, M. McLoughlin and A. Haimovitz-Friedman are at the Department of Radiation Oncology; M. Andreeff is at the Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, U.S.A.; and I. Vlodavsky is at the Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem, Israel.
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In the present study we compared the effects of BAEC/ECM and other substrata on the patterns of radiation dose survival curves of plateau phase BAEC. The data show that when assayed on top of their autologous ECM, BAEC exhibit a significantly broader threshold shoulder of their radiation dose survival curve as compared to cells plated on unrelated natural or on artificial substrata. This expression of improved capacity to repair radiation lesions, contingent upon interaction with specific micro-environmental factors of the culture system, stresses the need for the use of relevant experimental systems when extrapolations are made from *in vitro* data on radiation sensitivity of mammalian cells to predict their patterns of response *in vivo*.

MATERIALS AND METHODS

Cell cultures

Cloned populations of BAEC were established from the intima of bovine aorta as previously described [24]. Stock cultures were grown in 35 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose (1 g/l), 10% heat inactivated bovine calf serum (BCS), penicillin (50 U/ml) and streptomycin (50 μg/ml). 200 ng/ml of partially purified bovine brain bFGF (prepared as described previously [25]) or 10 ng/ml of purified recombinant bFGF (kindly provided by Dr J. Abraham, California Biotechnology, Inc.) were added every other day during the phase of exponential growth. After 8–10 days in culture, the cells reached confluence and exhibited features of contact inhibited monolayers. These plateau phase cells were either used for experiments, or were further subcultured (up to a maximum of 10 subcultures) at a split ratio of 1:10 to expand the cell population for other experiments. For subculturing, the monolayers were dissociated with 0.05% trypsin and 0.02% EDTA in phosphate buffered saline (PBS) (STV) (2–3 min at 22 or 4°C), washed twice in 10% BCS–DMEM and resuspended in DMEM with supplements as described above. These mild conditions of trypsinisation were sufficient to detach the cells, but did not injure, stimulate or affect cell functions in a detectable way. Cultures of the HR9-bFGF variant of the PF-HR9 mouse endodermal carcinoma cells [26] were grown in DME (4.5 g glucose/l) containing 10% FCS and the antibiotic supplements as described above. After reaching confluence, the cells were subcultured weekly at a split ratio of 1:10 (after dissociation with STV). All cell cultures were maintained at 37°C in 10% CO_2 humidified incubators.

Preparation of dishes coated with ECM

BAEC/ECM was produced as previously described [18, 19]. BAEC were seeded at an initial density of 2×10^4 cells per cm^2 and cultured in DMEM containing 10% BCS and 4% Dextran T-40. Partially purified bovine brain bFGF (200 ng/ml) or recombinant bFGF (10 ng/ml) was added every other day during the phase of exponential growth. 6–8 days after reaching confluency (12–14 days after the initiation of the culture) the cell monolayer was denuded by treatment with a solution containing 0.5% Triton X-100 and 20 mmol/l NH_4OH in PBS (3 min at 22°C), followed by 4 washes with PBS. While cellular debris and cytoskeleta of the lysed cells were effectively removed, the underlying ECM remained intact and adhered to the entire area of the tissue culture dish. The HR9-bFGF/ECM was prepared as previously described [26]. HR9-bFGF cells were seeded (10^5/35 mm dish) into culture dishes precoated with fibronectin (50 mg/dish) to enforce a firm adhesion of the secreted ECM to the plastic substratum. Ascorbic acid (50 mg/ml) was added on days 2 and 4. After 6–7 days in culture, the cell monolayer was denuded by treatment with a solution containing 0.5% Triton X-100 and 20 mmol/l NH_4OH in PBS (3 min at 22°C), followed by four washes with PBS. The ECMs could be stored in 4°C for several months before being used in experiments.

Irradiation of BAEC

24 h prior to irradiation the culture medium was changed into 10% BCS–DMEM. Immediately prior to irradiation, the cells were dissociated with STV, washed twice in 10% BCS–DMEM and resuspended in the same medium at a concentration of 10^5 cells/ml. Irradiation was carried out in a Gamma-cell 40 chamber containing two sources of ^{137}Cs (Atomic Energy of Canada) at a dose rate of 100 cGy/min. Single doses ranging from 200 cGy to 600 cGy were delivered, and the cells were plated immediately afterwards for colony formation tests. In some experiments the cells were seeded prior to irradiation in the tested types of culture dishes at the densities required for the colony formation assays, incubated for 60 min to permit a firm adhesion to the substratum tested, and irradiated *in situ*. The dose survival curves obtained with either of these two methods did not differ significantly.

Radiation dose survival curves

Survival after irradiation was defined as the ability of the cells to maintain clonogenic capacity and to form colonies. After irradiation the cells were plated for the colony formation assays in 35 mm dished (300–3000 cells/dish) with or without ECM coating. The medium consisted of DMEM and supplements as described above. Cells seeded on top of uncoated plastic surfaces received partially purified bovine brain bFGF (to a final concentration of 200 ng/ml) 16–18 h after irradiation, while exogenous bFGF was not added to cells cultured on ECM. In some experiments 10 ng/ml of purified recombinant bFGF was used instead of the partially purified brain bFGF, and similar results were obtained. After an additional 7–9 days in culture, colonies were fixed in 3.7% formaldehyde and stained with 1% crystal violet in ethanol. Colonies consisting of 50 cells or more were scored and 3–6 replicate dishes containing 50–150 colonies per dish were counted for each radiation dose tested. Survival curves were generated by a computer assisted program [27]. This program analyses the cellular survival after irradiation from raw data of colony counts of each culture dish included in an experiment. Using these data, the program calculates the surviving fractions for each dose point with associated weights, which are based on a Poisson distribution of the number of counts and the variation of colony counts for each data point. The program fits these data to the survival model tested (the single-hit multitarget model [28] or the linear quadratic model [29] by iteratively weighted least square regression analysis of all data points, estimating the covariance of the survival curve parameters and the corresponding confidence limits. The computer then plots the best fit survival curve and assigns a standard error bar to each data point, thus permitting statistical comparisons of corresponding points on different curves, and prints the computed Do, Dq and N number according to the single-hit multitarget model [28] and the α and β exponents according to the linear quadratic model [29].

^{3}H-thymidine labelling index

The fraction of cycling cells was evaluated by ^{3}H-thymidine labelling index as previously described. The cells were cultured in 10% BCS–DMEM and pulsed with ^{3}H-thymidine (9.25 kBq/well, 74 GBq/mmol). After 24 h of incubation the

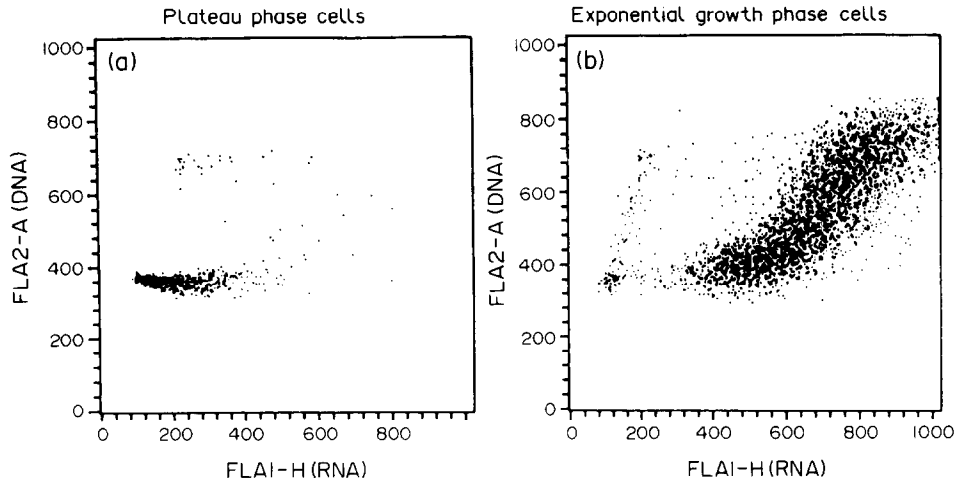

Fig. 1. Flow cytometry profiles of DNA and RNA in plateau phase (a) and exponentially growing (b) BAEC: Plateau phase cells were tested 9 days after reaching confluency (17 days after initial seeding), while exponentially growing cells were harvested and tested 5 days after initial seeding into culture. The staining for DNA and RNA was performed with acridine orange [30], and fluorescence measurements were scored on scattergrams by a single point for each cell representing simultaneously the staining intensities for both DNA and RNA.

medium was removed, the cells were washed five times with PBS, fixed with methanol for 10 min and dried out. The dishes were then incubated at room temperature in the dark, coated with Illford K5 photographic emulsion. After 5 days of incubation, the emulsion was developed with Kodak D-19 developer, fixed with 10% thiosulfate and stained with Giemsa. 1000 cells from each of two dishes were counted and the percentage of stained nuclei was determined.

Cell cycle analysis

The cell cycle distribution of BAEC was determined by flow cytometry after acridine orange staining of DNA and RNA as previously described [30]. Prior to staining the monolayers were dissociated with STV, washed twice with 10% BCS–DMEM and resuspended in PBS at a concentration of 2–4 $\times$ 10^5 cells/0.2 ml. The cells were then mixed with 0.4 ml of a solution containing 0.05 N HCl, 0.15 mol/l NaCl and 0.1% Triton X-100 (v/v). After 30 s, 1.2 ml of a solution containing 1m mol/l EDTA, 0.15 mol/l NaCl and 6 mg/ml of chromatographically purified acridine orange in 0.2 mol/l Na_2HPO_4/0.1 mol/l citric acid buffer (pH 6.0) was added. The cells were then passed through the focus of an excitation argon-ion laser beam (Lexel model 75), and the red fluorescence (F > 600) and green fluorescence (F = 530) emissions were measured for each cell by separate photomultipliers in a computer interfaced research cytofluorograph (modified model FC201, Ortho Instruments, Westwood, Massachusetts). The cytofluorograph was interfaced with a Hewlet-Packard microcomputer and the data were analysed using the "Cell Fit" software package (Becton and Dickinson) and presented via a Tektronix terminal.

RESULTS

Cell cycle distribution of plateau phase BAEC

The dose survival experiments carried out in this study were performed with plateau phase BAEC, 7–9 days after reaching confluency. The cell cycle distribution of these cells, determined by flow cytometry after acridine orange staining of DNA and RNA, is shown in Fig. 1a. Data pooled from six experiments showed that 91 (1.13)% [mean (S.E.)] of the cells were in G_0–G_1 phase, 3.66 (0.71) in S phase and 5.33 (0.66)% in G_2–M phase. Analysis of the total cellular RNA content indicated that the majority of the G_0–G_1 cells were in fact in the low RNA G_0 state (Fig. 1a). For comparison, the profiles of exponentially growing BAEC are also shown (Fig. 1b). The percentage of G_0–G_1 cells was 46.2 (0.25)%, while 45.5 (0.64)% were in S phase and 8.3 (0.48)% in G_2–M phase. ^{3}H-thymidine labelling and autoradiography showed that less than 6% of the plateau phase cells exhibited staining of nuclei, consistent with the cell cycle profiles obtained by the flow cytometry method.

Clonal growth characteristics of BAEC on ECM and plastic

The clonal growth patterns of BAEC varied according to the culture conditions and the substratum used for the colony formation assays. When 400 cells were plated on top of uncoated plastic of standard culture dishes in the absence of bFGF in the medium, there were no detectable colonies after 7–10 days in culture. Addition of 200 ng/ml of partially purified bovine brain bFGF or 10 ng/ml of purified recombinant bFGF concomitant with cell seeding resulted in colony growth stimulation, with plating efficiencies of 10–30% in five consecutive experiments was 17.3 (5.2)%. Delay in addition of bFGF up to 24 h after seeding did not affect the plating efficiency of cells assayed on plastic, but further delay was associated with cell senescece and decreasing cloning efficiency. Dose-response experiments showed a direct relationship between the medium concentration of bFGF and its effect on the plating efficiency on plastic, with 200 ng/ml of the partially purified bFGF being within the plateau range for this effect (Table 1).

In contrast to cultures on uncoated plastic, when BAEC were seeded at clonal densities on top of BAEC/ECM or HR9-bFGF/ECM, colonies were detected 7–9 days later without requiring the addition of exogenous bFGF. This is consistent with the fact that both BAEC/ECM and HR9-bFGF/ECM were shown to contain biologically active matrix-bound bFGF [26]. When cultured on BAEC/ECM, the mean plating efficiency in the present series of survival curves was 20.6 (6)% (range 10–23%) (Table 2), although in other experiments even higher values were observed (Table 1). Addition of 200 ng/ml of partially purified bFGF to the medium of unirradiated cells cultured on top of the BAEC/ECM did not result in a further increase of the cloning efficiency (Table 1). Similarly, the plating efficiency on HR9-bFGF/ECM was 19.4 (3.7)% (Table 2) and it was not

Table 1. Effect of bFGF on cloning efficiency of plateau phase BEAC cultured in ECM coated dishes or on regular tissue culture plastic

Type of substratum	bFGF (ng/ml)	Cloning efficiency (%)
Plastic	0	0
	25	13.7
	50	19.0
	100	20.0
	200	19.0
	400	20.5
HR9-	0	23.9
bFGF/ECM	200	23.0
BAEC/ECM	0	47.1
	200	42.0

400 cells were seeded into 35 mm culture dishes with or without ECM precoating. The number of colonies was scored 9 days later. Each data point represents the mean of five dishes.

affected by addition of 200 ng/ml of partially purified bFGF (Table 1).

Radiation dose survival curves of plateau phase BAEC

Figure 2 shows radiation dose survival curves generated with plateau phase BAEC, assayed immediately after irradiation under three different culture conditions. Some of the assays were performed in standard culture dishes (on top of uncoated plastic surfaces), with the cells receiving 200 ng/ml partially purified bovine brain bFGF 16–18 h after irradiation, while other assays were carried out on top of BAEC/ECM or HR9-bFGF/ECM, without addition of exogenous bFGF. As shown in Fig. 2, the slopes of the dose–survival curves did not differ significantly, but the shoulder regions were significantly different, depending on the type of substratum used. Table 2 summarises the parameters of these dose survival curves pooled from three experiments. The Do on plastic, calculated according to the single-hit multitarget model [28], was 107 (6) cGy, compared with 106 (2) cGy on BAEC/ECM and 108 (8) on HR9-bFGF/ECM. These differences were statistically not significant. On the other hand, the Dq on plastic [63 (28) cGy] was significantly decreased compared with the Dq on BAEC/ECM [194 (8) cGy; $P < 0.05$]. When instead of the autologous BAEC/ECM, the natural but biologically unrelated HR9-bFGF/ECM was used, the Dq value [156 (30) cGy] was significantly different ($P > 0.05$) compared with those observed on either plastic or BAEC/ECM, but the Do [106 (2)] was not significantly different from the values observed on either of the other substrata. The decrease in Dq on HR9-bFGF/ECM compared with BAEC/ECM did not result from suboptimal quantities of bFGF bound to this matrix, since addition of 200 ng/ml of partially purified bFGF to the media of the cells assayed on HR9-bFGF/ECM did not change the parameters of the dose survival curves (Fig. 3). Similarly, addition of bFGF to cells assayed on BAEC/ECM did not affect the pattern of the dose survival curve on this matrix (data not shown).

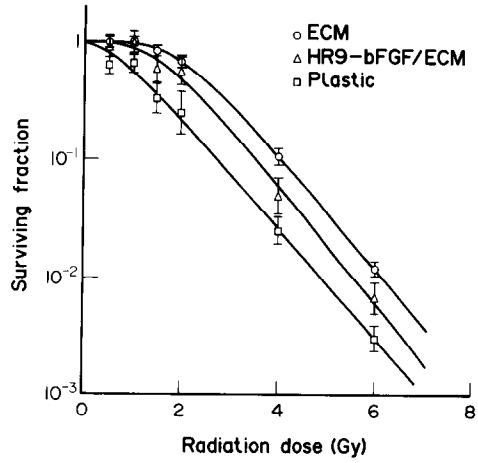

Fig. 2. Radiation dose survival curves of plateau phase BAEC plated for colony formation assays on either ECM or on regular tissue culture plastic: The cells were irradiated in suspension (10^5 cells/ml) and seeded for colony formation assays in standard tissue culture dishes (△) (on top on the uncoated plastic surfaces), or in culture dishes precoated with either the BAEC/ECM (○) or with the HR9-bFGF/ECM (□). The cells plated on plastic received partially purified bovine brain bFGF (200 ng/ml) 18 h after seeding, while the cells seeded on either of the ECMs did not receive exogenous bFGF. The survival curves shown represent the best fit curves calculated by a computerised program [27] according to the single-hit multitarget model [28] and plotted by the computer as described in the Materials and Methods. Each data point represents the mean (S.E.) of 6–8 dishes.

The sensitivity of the dose–survival relationship at the low dose range to the type of substratum used in the colony formation assay was also observed when the data were analysed according to the linear quadratic model [29]. Table 2 shows that the α and β exponents differed significantly according to the substrata upon which the cells were cultured after irradiation. Plating the cells on BAEC/ECM was associated with the lowest α values [0.059 (0.007) Gy^{-1}] and the highest β values [0.101 (0.01) Gy^{-2}], with significant increases in α values and decreases of β values on the HR9-bFGF/ECM and plastic.

Table 2. Parameters of radiation dose survival curves of plateau phase BEAC plated for colony formation assays on ECM or on regular tissue culture plastic

	Plastic	HR9-bFGF/ECM	BAEC/ECM
Plating efficiency (%)	17.3 (5.2)	19.4 (3.7)	20.6 (6.0)
Do (cGy)	107 (6)	108 (8)	106 (2)
Dq (cGy)	63 (28)	156 (30)	194 (8)
n Number	1.8 (0.52)	4.2 (1.6)	6.2 (0.7)
α (Gy^{-1})	0.583 (0.09)	0.181 (0.05)	0.059 (0.07)
β (Gy^{-2})	0.044 (0.02)	0.069 (0.03)	0.101 (0.01)

The data were pooled from three experiments. The values of Do, Dq and *n* were computed using the single-hit multitarget model [28], and the α and β exponents were calculated using the linear quadratic model [29]. The data are presented as the mean (S.E.).

DISCUSSION

Our data demonstrate that the patterns of response of endothelial cells to irradiation *in vitro* are significantly affected by the microenvironmental conditions under which the experiments are carried out. The data suggest that the capacity of endothelial cells to recover from radiation damage *in vitro*, as expressed by parameters of the dose survival relationship, is dependent upon several specific postradiation culture factors. In a recent study

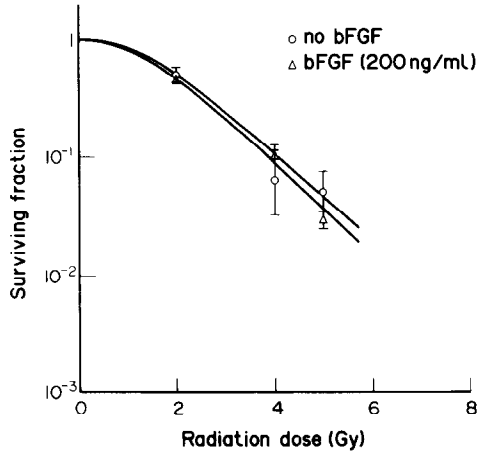

Fig. 3. Effect of bFGF on the radiation dose survival curves of plateau phase BAEC plated for colony formation assays on the HR9-bFGF/ECM. The cells were irradiated and plated into HR9-bFGF/ECM coated culture dishes as described in Fig. 2. Partially purified bovine brain bFGF (200 ng/ml) was added at 0'time to some of the cultures as indicated. The differences in Do (114 and 109 cGy) and Dq (151 and 142 cGy) were not significant.

we showed that bFGF serves as a potent inducer of potentially lethal radiation damage repair (PLDR) in BAEC, and also stimulates the generation of a threshold shoulder in the dose survival curves [31]. The present dose survival experiments were in fact carried out under bFGF stimulation. Although the media on the BAEC/ECM or the HR9-bFGF/ECM cultures were not supplemented with bFGF, these culture systems still exposed the irradiated cells to bFGF stimulation, since our recent studies showed that both types of ECM contained biologically active heparan sulphate-bound bFGF [19, 23, 26]. The dependence of Dq, n, α and β on the type of the culture substratum suggests that in addition to the effects of bFGF, interactions of irradiated cells with components of the basement membrane upon which they rest also affect their repair capacity of radiation lesions. Furthermore, the improved repair on BAEC/ECM suggests that the repair function is most optimally expressed upon interaction of irradiated cells autologous ECM. The possibility that the decreased repair observed on HR9-bFGF/ECM resulted from low quantities of matrix-bound bFGF was excluded by the experiment which failed to show a change in the shape and parameters of the dose survival curves when exogenous bFGF was added to the culture medium (Fig. 3). Kofstad and Sutherland [32] recently showed that the radiosensitivity of human ovarian carcinoma cell lines cultured on Matrigel ECM was not different from the sensitivity of cells assayed on plastic. It should, however, be noted that Matrigel is a reconstituted basement membrane-like gel [33], which represents a mixture of ECM components extracted from the mouse EHS tumour that spontaneously form a stable gel at 38°C. Being of mouse origin, the Matrigel is xenogeneic to human ovarian carcinoma cells. Indeed, our experiments showed that the xenogeneic combination of BAEC and the mouse HR9-bFGF/ECM exhibited radiosensitivity characteristics not significantly different from those observed on plastic. It seems, therefore, that the substratum upon which irradiated endothelial cells rest is not only capable of modulating their repair capacity of radiation lesions, but that there is a high sensitivity to specific matrix components in terms of expression of metabolic functions involved in the repair.

The sensitivity and specificity of the repair function to components of autologous ECM are consistent with other known effects of matrix on mammalian cells. The tissue matrix is composed of basement membranes and the ground substance of the stroma, and its tissue specificity relates to variations in the supramolecular assembly of collagens, elastin, structural glycoproteins (i.e. laminin, fibronectin, entactin, nidogen, thromboplastin) and proteoglycans [34]. Anchorage of cells to basement membranes is mediated via focal adhesion plaques that bind the cell membrane to specific domains of ECM molecules and also serve as structural links between the ECM and the cytoskeleton [35]. These highly specialised structures of the plasma membrane contain in addition to adhesion molecules also several regulatory proteins that mediate transmission of ECM stimuli to the cytoplasm by chemical signaling similar to that triggered by soluble growth factors [36–38]. Adhesion plaques seem, therefore, to be involved not only in binding of cells to ECM, but also in mediating specific cellular functions. ECM-cellular linkages were indeed found to control both the shape, spatial orientation and tissue distribution of normal cells [18, 20] and to affect cytoskeletal organisation [39]. In addition, they were shown to regulate cytodifferentiation and cell migration during embryogenesis [21], to induce the expression of differentiation genes in mesenchymal and epithelial cells [20, 21, 40–42], and to modulate the response of cells to hormones and growth factors [19, 26, 43]. Whereas our previous study showed that bFGF serves as a potent inducer of radiation damage repair in endothelial cells [31], our present data demonstrate an ability of ECM to modulate this function. Improved repair, however, was observed only when the cells were plated on top of their autologous BAEC/ECM and not on the xenogeneic HR9-bFGF/ECM. The specificity of the repair function to components of autologous matrix is consistent with our previous data which showed that plating BAEC on their autologous BAEC/ECM promoted both proliferation and expression of normal differentiation functions, while plating on ECM produced by human fibroblasts induced proliferation but resulted in loss of characteristic differentiation functions [44]. The BAEC/ECM and the HR9-bFGF/ECM were shown to differ in composition and structure [45] and to confer different biological responses in cells seeded on top of them [46]. Whether the improved repair of radiation damage of BAEC/ECM results from optimal cell membrane receptor distribution that facilitates the targeting of ECM-bound bFGF to its specific cell surface receptors, or whether there is synergism between signals transduced from bFGF stimulation and signals transduced from stimulation by specific molecular sequences found in the BAEC/ECM, but not in the HR9-bFGF/ECM, is unknown.

Whether bFGF and matrix components are directly involved in expression and/or modulation of the response of endothelial cells to irradiation *in vivo* is unknown. Similar to the plateau phase cells used in the present study, vascular endothelial cells *in vivo* are mostly quiescent, and exhibit a low incidence of cycling cells with labelling index values of less than 1% and estimated doubling time of 50–1000 days [47]. After irradiation there is only a minimal increase in the proliferative activity up to a doubling of the labelling index [48]. Nevertheless, the clonogenic capacity of endothelial cells *in vivo* can be evaluated if angiogenic stimuli are produced. Physical or chemical stimulation of the rat subcutaneous air pouch or of the dog and rabbit cornea result in capillary sprouts, each considered a clone of endothelial cells [49–51]. Within 7–12 days, a network of neovascular capillaries is produced, the density of which is considered proportional to the clonogenic capacity of the stimu-

lated endothelial cells. Using such techniques, radiation dose-effect curves showed slopes with Do of 170–265 cGy, and split dose experiments resulted in D_2–D_1 values of 180–290 cGy [49–51]. Whereas it is unknown whether FGF is involved in the production of the wide shoulders observed in these experiments, it should be noted that FGF is known to be a potent angiogenic factor that stimulates neovascularisation *in vivo* [52], and is likely to be an active participant in these experimental systems. Of particular interest and possible relevance are also our recent immunohistochemical studies which showed that bFGF is ubiquitously detected in the basement membranes of all size blood vessels, although the intensity and patterns of localisation varied among different regions of the vascular bed [53]. Whereas homogeneous and intensive immunoreactivities were observed in large and intermediate size blood vessels, heterogeneity of expression was found in capillaries. The most intense capillary immunoreactivity was observed in the basement membrane sites of branching points of mid-size capillary vessels, while staining became progressively less intense in nonbranching portions of capillaries and seemed to be absent in some sections [53]. The variations in detectability of bFGF in vascular basement membranes correlate with reported sensitivities of different sections of the vascular system to irradiation *in vivo*. Ultrastructural studies indicated that capillaries represent the most radiation sensitive sections of the vascular system [1–4]. Electron microscopy studies of the irradiated rabbit myocardium performed by Fajardo [9] showed that whereas the endothelium of capillaries exhibited severe changes of radiation damage, endothelial cells of large vessels and of the endocardium, even when present in the same tissue sections, seemed to be intact.

It seems, therefore, that the BAEC/ECM model stimulates conditions that prevail in the vascular intima *in vivo*, and that this experimental system may be a relevant model for the study of factors that modulate the radiation response of endothelial cells *in vivo*. Establishment of similar criteria of relevance may be critical when projections are made from parameters of response of mammalian cells to irradiation *in vitro* to their expected radiosensitivity *in vivo*. Several authors have recently demonstrated considerable variations in radiosensitivity among human tumour cell lines and suggested that the established degrees of sensitivity *in vitro* correlate with the response of these tumours to irradiation *in vivo* [54–57]. Other investigators have been unable to consistently demonstrate such correlations [58–60]. Since none of the reported studies have used autologous matrix substrata, and since in most instances there was no use of specific media supplements as may be required by some cell types, the specificity and relevance of observed correlations could, at least in some cases, be questioned. Whether mammalian cells other than BAEC also exhibit sensitivity of their *in vitro* radiation response to specific microenvironmental factors remains to be studied. However, the possibility that such phenomena do exist and may affect patterns of the dose–survival relationship should be considered whenever projections are made from *in vitro* data to expected patterns of response of mammalian cells to irradiation *in vivo*, such as has been recently suggested by several predictive assay systems for clinical response to radiation in human tumours.

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