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ORIGINAL ARTICLE

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Repopulation of osteosarcoma cells after treatment with doxorubicin in the presence of extracellular matrix biopolymers

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Abstract *Purpose*: To elucidate the role of extracellular matrix (ECM) in repopulation capacity of osteosarcoma cells after doxorubicin treatment. *Methods*: OSCORT cells established in our laboratory from a human osteosarcoma, were treated with doxorubicin in monolayer for 4 h, then cells were further incubated either in monolayer or in ECM-containing three-dimensional cell-culture (3-DCC), apoptosis induction and changes in cell number were measured. Alkaline comet assay was applied to estimate DNA damage, immunoblot technique and immunocytochemistry were used to investigate p53 protein synthesis, and the repopulating capacity in monolayer culture and in ECM-based 3-DCC, after doxorubicin treatment was measured. In addition to OSCORT culture five other human cell lines (HT-1080, PC-3, MDA-MB231, A-431 and ZR-75-1) were used to compare the antimigratory and antiproliferative effects of doxorubicin. *Results*: The apoptotic index, the extent of DNA damage and the representation of p53 were much lower in the OSCORT cell cultures if the cells were exposed to ECM after treatment with doxorubicin. The doxorubicin-treated OSCORT cells transferred from the monolayer culture were not able to proliferate at all, at the same time, the cytoprotection provided by ECM prevailed upon transferring the cells into plastic dish, and resulted in potent repopulation capacity of the cells. *Conclusions*: Present data indicate that ECM contributes to failure in therapy of human osteosarcoma in clinical situation. Overall, the application of ECM-based 3-DCC could be suggested as an appropriate model system for the better understanding of antitumor drug action and hereby to set the stage for promising novel pharmacological approaches in cancer therapy.

Keywords Osteosarcoma · Cytoprotection · Extracellular matrix · Doxorubicin · Repopulation · Proliferation · Migration

Abbreviations ECM: Extracellular matrix · EHS-ECM: Extracellular matrix isolated from Engelbreth–Holm–Swarm sarcoma · OSCORT-ECM: Extracellular matrix prepared from OSCORT cell culture · 3-DCC: Three-dimensional cell-culture · FCS: Fetal calf serum · HSPG: Heparan sulfate proteoglycan · GF: Growth factor · Dox: Doxorubicin · MMP: Matrix metalloproteinase

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Introduction

Osteosarcoma, the most common primary malignant bone tumor, is characterized by an extremely aggressive clinical course. The introduction of neoadjuvant and adjuvant chemotherapy markedly improved the outcome with long-term relapse-free survival rates ranging from 55 to 75%, however, approximately 30–40% of patients will relapse, most often with pulmonary metastases [1, 11, 18]. Patients whose tumors respond poorly to chemotherapy are at a higher risk of relapse, therefore it is most desirable to elucidate and abrogate the limiting factors which contribute to failure in drug therapy [2].

Recently, the role of extracellular matrix (ECM) as modulator of antitumor drug action has also been suggested [6, 15, 19]. The ECM consists of a network of collagens, laminin, proteoglycans, fibronectin and other

biopolymers; it has multiple functions both in physiological and pathological conditions, i.e., maintenance of organ structure, controlling organ-specific cell functions, offering substrate for cell migration, storage of growth factors, initiating signals towards gene expressions [7, 9, 12, 13]. In addition, several lines of experimental data including ours provided evidence that doxorubicin, etoposide and hydroxy-cyclophosphamide-induced cell death could be suppressed in the presence of ECM [6, 17, 19]. As several studies concluded that the pathobiological events in tumor progression are guided by ECM, it seemed an appropriate question: whether ECM could provide protection against the effects of doxorubicin and contribute to the repopulation of tumor cells following treatments.

Materials and methods

Chemicals

Cell culture media reagents and chemicals of analytical grade were purchased from Sigma (St. Louis, MO, USA); Merck (Darmstadt, Germany); Boehringer Ingelheim (Heidelberg, Germany) and Boehringer Mannheim (Mannheim, Germany). Doxorubicin was provided by Pfizer Ltd. (Budapest, Hungary). The p53 antibody is specified in the corresponding sections.

Cell cultures

Human osteosarcoma cell culture (OSCORT culture) was established from a primary osteosarcoma of a 17-year-old male as described previously [6, 7]. The human tumor cell lines (HT-1080 fibrosarcoma, PC-3 prostata carcinoma, A-431 epidermoid carcinoma, MDA-MB-231 and ZR-75-1 mammary adenocarcinoma were kindly provided by Professor József Timár (National Institute of Oncology, Budapest, Hungary) and Professor Rosita Winkler (University of Liege, Belgium).

Cells were cultured in RPMI-1640 medium + 10% fetal calf serum, supplemented with penicillin (100 U/ml medium), streptomycin (100 μg/ml medium), at 37°C, in a humidified 5% CO_2 atmosphere.

Preparation of extracellular matrix and isolation of its biopolymers

Extracellular matrix was isolated from Engelbreth–Holm–Swarm (EHS) sarcoma (EHS-ECM, ECM gel) as described by Kramer et al. [10], ECM was also prepared from OSCORT cell culture (OSCORT-ECM) as described previously [6, 7]. The ECM biopolymers were prepared from EHS-ECM (laminin, collagen type IV and perlecan) crude extract as reported by Kleinman et al. [9] and Lyon et al. [13]. In some studies, growth factor-poor ammoniumsulfate-fractionated EHS-ECM was utilized following the method of Taub et al. [25] and further purified by membrane filtration. Centriplus Centrifugal Filter Units (Millipore) were used with nominal molecular weight limits of 100,000 and 10,000 kDa according to the user guide of the producer. The purity of ECM components was analyzed by SDS-PAGE and Coomassie R 250 gel staining (Serva, Heidelberg, Germany). Fibronectin from human plasma was purchased from Sigma. Composition of EHS-ECM isolated in our laboratory was reported previously [7, 17].

Measurements of doxorubicin action on cell culture

For cell proliferation, viability, protein expression, DNA damage and flow cytometric measurements 7×10^4 cells/cm^2 were plated on 24-well plates (Greiner, Nürtingen, Germany). Forty-eight hours after plating monolayer cultures were treated with doxorubicin at the indicated concentration, after that, cells were washed twice with RPMI-1640 medium and further cultured either on plastic substrate or in extracellular matrix gel (ECM gel) (i.e., monolayer cultures overlaid with 10 mg/ml ECM in RPMI-1640 medium + 10% fetal calf serum ECM) and allowed to grow for additional 24–120 h. Alternatively, the medium was supplied with purified ECM components at protein concentrations of 10, 50 or 100 μg/ml. After incubation for the indicated time the ECM was digested (2.5 mg/ml dispase for 20 min), the released cells combined with the monolayer were counted and processed for further studies.

To investigate changes in tumor cell proliferation and migration 10^5 tumor cells were plated and 24 h later treated with doxorubicin at the indicated concentrations for 4 h. Then doxorubicin was removed, fresh medium with or without ECM gel as described previously was added and cells were incubated in the three-dimensional cell-culture (3-DCC) for 24 h. For cell counting the migrating cells in the ECM gel were released by gentle mechanical stirring, whereas the nonmigrating cells were released from the monolayer with trypsin-EDTA.

To measure the action of ECM biopolymers on *invasive growth* osteosarcoma cells were cultured as monolayers for 48 h in the presence of 50 μg/ml of ECM biopolymers, then 0.75% agarose gel was overlaid on them, and the tumor cells were let to migrate into the agarose gel in the next 24 h. At the end of the 24 h incubation the agarose gel with the migrated cells was mechanically separated from the monolayer, and the number of cells from both compartments were counted.

To determine *repopulation capacity* OSCORT cells, treated with doxorubicin or untreated, were grown either as monolayer culture or in ECM-based 3-DCC for 3 days then transferred to plastic dish applying 7×10^4 viable cells (measured by trypan-blue exclusion test) as inoculum.

Cell proliferation was measured by cell counting in hemocytometer or by applying succinate dehydrogenase test (MTT-assay) [6, 16].

Cellular viability was tested with trypan-blue exclusion test, while the *apoptotic index* was determined as reported before; by using combined staining with acridine orange and ethidium bromide [17].

Flow-cytometry technique was used to measure *cell population kinetics*. Cell cycle parameters were studied as reported previously [6, 17].

Immunoblot analysis

Cells from monolayer and 3-DCC cultures were released as described previously. Equal number of cells (10^5) were lysed in Laemmli's, buffer and proteins were separated in sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to nitrocellulose membranes (Bio Rad, Hercules, CA, USA) and then reacted with p53 mouse monoclonal antibody (clone DO-7) from DAKO (Glostrup, Denmark) used in dilutions 1:1,000 [5]. Immunoblots were developed with 3,3′ diamino-benzidine tetrahydrochloride (Sigma) and densitometred with Eagle Eye II still video system (Stratagene, La Jolla, CA, USA).

Immunocytochemistry

After application of cytospin (Shandon, Pittsburgh, PA, USA), cells were fixed for 15 min with methanol and for 3 s with acetone, blocked with 5% bovine serum albumin in PBS. Primary anti human-p53 antibody was used overnight in 1:200 dilutions. Primary antibodies were detected by biotinylated anti-mouse or anti-rabbit secondary antibodies (Vector, Burlingame, CA, USA) and streptavidin–fluorescein (Amersham, Buckinghamshire, UK). Antibody reactions were analyzed in a laser-scanning microscope (Bio-Rad, Hercules, CA, USA).

Analysis of DNA damage

For spectrofluorometric quantitation of DNA fragments following exposure to doxorubicin the treated and the untreated cells were washed in PBS and resuspended in lysis buffer (0.05% fully reduced Triton X-100, 5 mM Tris, 20 mM EDTA pH 8). Cell lysates were centrifuged at 20,000×*g* for 25 min at 4°C to separate fragmented DNA from intact chromatin. DNA in both compartments was stained with bisbenzimide H 33342 (1 μg/ml) and quantified by using DNA Quant. 2OOO fluorimeter, (Hoefer, San Francisco, CA, USA) [14].

DNA breaks were measured also by using alkaline single cell gel electrophoresis (comet) assay [22]. All procedures were carried out on ice and in subdued lighting. The cell samples (10^4/10 μl) were mixed with 90 μl of 1% low melting point agarose and spread onto frosted microscope slides covered with a thin layer of 0.6% normal melting agarose. A top layer of 0.5% low melting point agarose was used to protect the cells. Cells were lysed by 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton-X 100 and 1% sodium sarcosinate (pH 10) at 4°C overnight. After lysis the slides were immersed in alkaline electrophoresis buffer (300 mM NaOH, 10 mM EDTA, pH 13) for 60 min at 25°C in the dark with two buffer changes to unwind the DNA. The slides were then subjected to electrophoresis in the same buffer for 20 min at 4°C and 4 V/cm. The cells in the slides were stained with ethidium bromide (1 μg/ml) and the images of comets were acquired with a Zeiss Axioskop fluorescent microscope (Zeiss, Jena, Germany), amplified, recorded and fed into a multiparameter image analysis program (IMAN morphometric software KFKI Hungary, 1997). The quantitative parameters of the comet assay took the size and density of the head and tail into consideration. The tail length (l) gave the length of the tail without the head, which was related to the size of the DNA fragments, since the smaller fragments moved further in the gel. The tail percent (T) was the density of the tail related to the density of the head, expressed in percent, which correlated with the quantity of the fragmented DNA. The "tail-moment" (M) was calculated as the product of the tail length and the percentage of DNA in the tail; [$M = (T \times l)/100$], representing both the extent of DNA fragmentation and its severity [22].

Statistical analysis

Results were expressed as means ± SD and statistical analysis were performed using the Graphpad Prism Software. Values giving a probability of less than 5% were considered as significant (i.e., $P < 0.05$).

Results

Proliferation and migration of OSCORT cells in the presence of ECM biopolymers

The growth modulatory action of soluble ECM and also of its components at concentrations between 10 and 100 μg/ml were described previously ([7], Table 1). In consistence with the already published data [7], growth of OSCORT cell culture was stimulated both by ECM isolated from EHS tumor (EHS-ECM, ECM gel) and ECM secreted by OSCORT cells (OSCORT-ECM). In order to further prove that the stimulation of cell growth was due to the ECM itself and not due to *other components* like *growth factors, cytokines* that often attach to the ECM, and are isolated together with it, *growth factor-reduced EHS-ECM* was prepared by additional purification steps [25]. The growth factor-reduced EHS-ECM has shown similar effects as the original EHS-ECM (Table 1). It is worthy of note the difference in the contribution of each ECM components to this growth stimulation: laminin as the major ECM component (80% of total proteins in EHS-ECM) showed no effect,

Table 1 ECM biopolymers as modifiers of cell growth and doxorubicin action

ECM biopolymer (50 μg/ml)	Untreated culture		Treated culture
	Change in growth (% of control)	Distribution of cells in S-phase (%)	0.5 μg/ml doxorubicin action (% of untreated culture)
No biopolymer (control)	100 ± 3.2	14.90 ± 1.7	58 ± 6.0*
Perlecan	159 ± 6.0*	32.00 ± 1.4*	85 ± 3.8
Fibronectin	154 ± 1.0*	22.20 ± 0.7*	85 ± 5.2
Laminin	100 ± 7.1	16.90 ± 2.4	63 ± 4.1*
Collagen type IV	67 ± 5.7*	9.80 ± 2.3	76 ± 3.5*
GF-reduced EHS-ECM	147 ± 4.0*	28.31 ± 0.8*	85 ± 2.6
EHS-ECM	147 ± 6.0*	29.73 ± 1.5*	86 ± 3.7
OSCORT-ECM	156 ± 5.2*	24.53 ± 2.2*	85 ± 4.0

Above shown are changes in cell culture growth and also the distribution of cells among cell cycle phases in the presence of 50 μg/ml ECM biopolymers. Results represent the mean ± SD of three independent experiments
*Significantly different from the corresponding control group: ($P < 0.05$)

collagen type IV reduced, but fibronectin and perlecan stimulated the cell culture growth (Table 1). As it was described before [7], at the same stage of the growth of OSCORT culture the percent of cells in S-phase was elevated in the presence of ECM, perlecan or fibronectin relative to the untreated cultures, while the cell cycle was not affected by laminin ([7], Table 1). In contrast, collagen type IV induced an accumulation of cells in G2-phase [7].

It was further proved that the ECM components elicited changes not only in the size of the OSCORT cell population, but also in their migratory capacity; a remarkable increase was recorded in the migratory compartment of the 3-DCC if the cells were exposed to perlecan or fibronectin ([7], Table 2). Interestingly, collagen type IV although reduced culture growth, elicited a modest increase in the number of migrating cells (Table 2).

Induction of apoptosis, DNA damage and expression of p53 in doxorubicin-treated OSCORT cells grown in ECM gel

The previously illustrated data indicating that proliferation and migration of OSCORT cells could be stimulated by ECM biopolymers have given reason to investigate cellular responses to doxorubicin action in the presence of ECM. Figure 1 shows the reduced frequency of apoptosis in OSCORT cell population growing in 3-DCC containing ECM gel relative to cells in monolayer culture both pretreated under the same circumstances with 0.1, 0.5, 5 and 10 μg/ml doxorubicin.

Single-cell gel electrophoresis assay was used to detect doxorubicin-induced DNA breaks in OSCORT cells grown in monolayer culture or in 3-DCC containing EHS-ECM. Figure 2 shows induction of tail formation of the cells (hence this technique is designated as comet assay) after treatment with doxorubicin, it is noteworthy however, that its severity was dependent on the post-treatment conditions. In the cell population exposed to ECM gel there were much less cells found with comet formation representing DNA breaks. Parameters for the alkaline comet assay offer quantitative measurements on the incidence and length of DNA fragments in cells treated with doxorubicin (5 μg/ml) as follows: the denaturing condition (> 12 pH) fragmented 89.7 ± 4.3% of the DNA in cells grown in monolayer culture after doxorubicin treatment, however this value was only 52.7 ± 4.3% in cells exposed to ECM. The length of doxorubicin-induced fragments was also reduced in 3-DCC, which is illustrated by the extensive increase of the comet length from 18.1 ± 2.3 μm control value to 37.5 μm in doxorubicin-treated cells from the monolayer culture. On the contrary, in cells exposed to ECM the control value increased from 15.7 μm only to 21.85 μm. The "tail moment" including both the size and quantity of the fragments indicates the difference of the severity of DNA damage in cells grown in the presence or absence of ECM following doxorubicin treatment. Thus, in cells from the monolayer cultures the control value for the tail moment was 5.8 ± 1.4, which increased to 34.0 ± 1.9 after treatment with 5 μg/ml doxorubicin. It is

Table 2 Modification of OSCORT cell migration in 3D-agarose cell culture by ECM biopolymers

	ECM biopolymer (50 μg/ml)				
	Control	Perlecan	Fibronectin	Laminin	Collagen IV
Invasive index[a] (%)	20 ± 0.2	53 ± 0.5*	32 ± 0.8*	20 ± 0.8	32 ± 0.4*

Results represent the mean ± SD of three independent experiments
*Significantly different from the corresponding control group: ($P < 0.05$)
[a]Invasive index: migrated cells/total number of cells × 100

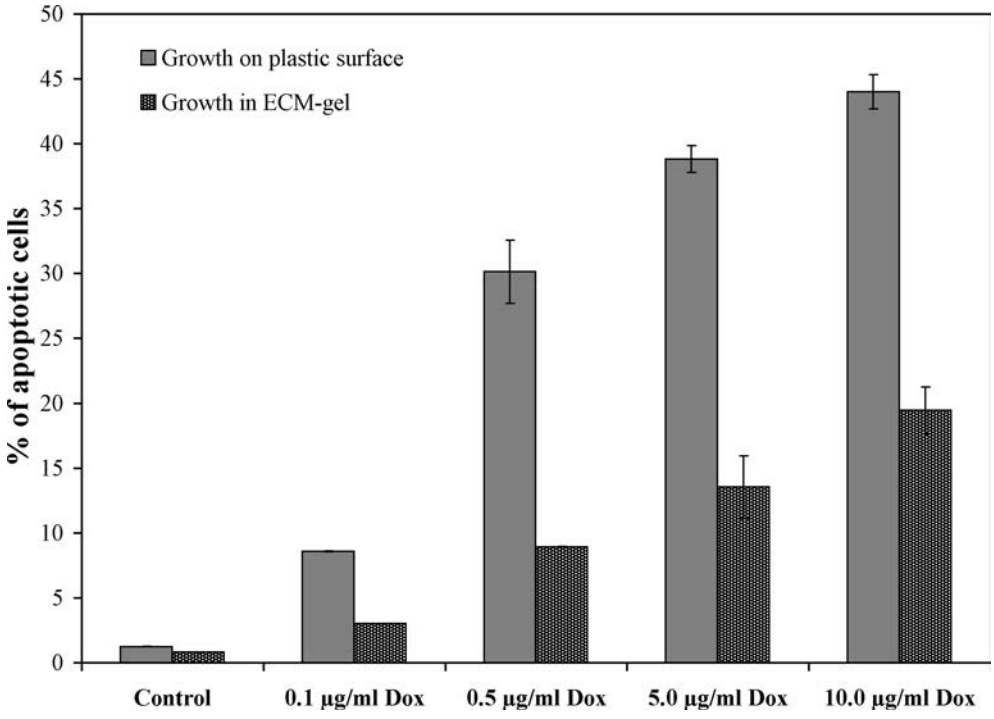

Fig. 1 Doxorubicin induction of apoptosis modified by ECM. The effects of 0.1, 0.5, 5 and 10 µg/ml doxorubicin on apoptosis of OSCORT cells cultured on plastic surface or in ECM gel were compared using combined staining with acridine orange and ethidium bromide. The cells were uniformly treated on plastic for 4 h, then they were cultured either on plastic surface or in ECM gel for 72 h. In the cell cultures on plastic surface the doxorubicin induced more apoptosis. The *bars* represent the mean ± SD of three independent experiments

interesting, that in cells isolated from the 3-DCC the same treatment resulted in a much modest level of DNA damage i.e., the 4.6 ± 1.3 control value increased only to 11.4 ± 2.5 (Fig. 2).

The induction of DNA fragments by doxorubicin was also detected by separating fragmanted and chromatin DNA under neutral conditions. At the end of the 4 h doxorubicin treatment, 12–15% of total DNA could be recovered as fragments, which value increased to 27% if the cells were further grown in the monolayer culture, but virtually no change was recognized in the 3-DCC model 24 and 48 h later (data not shown).

As DNA damage elicits activation of p53, it was investigated if the OSCORT cells grown in monolayer culture or in the presence of EHS-ECM show similar levels of p53 expression after doxorubicin treatment. By application of immunoblot technique lower level of p53 was measured in untreated cells grown in 3-DCC relative to cells in monolayer culture. Following the treatment with 5 µg/ml doxorubicin, the p53 level increased by threefold in monolayer cell culture but only by 66% in cells exposed to ECM. Immunocytochemical studies illustrated the difference between p53 synthesis of OSCORT cells grown in monolayer and in 3-DCC (Fig. 3).

Repopulation of OSCORT cells after treatment with doxorubicin

Having shown the protective effect of ECM against apoptosis and DNA damage induced by doxorubicin,

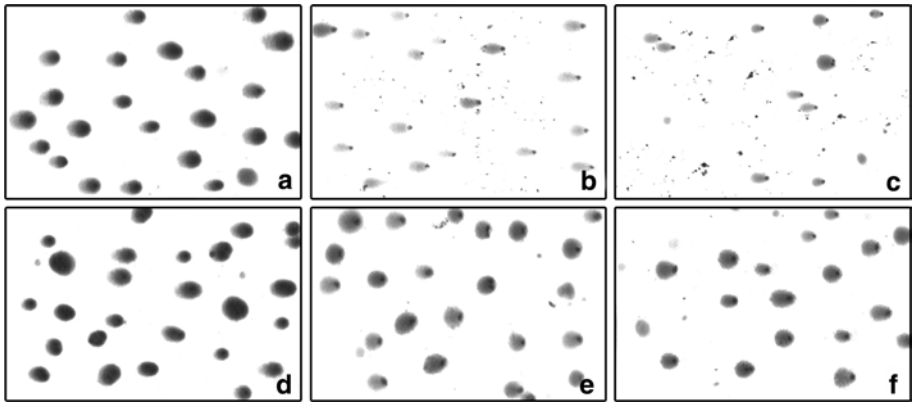

Fig. 2 Detection of DNA damage using comet assay at 72 h after treatment as described in Materials and methods. **a** Control cells from monolayer, **b** cells treated with 0.5 µg/ml doxorubicin and cultured as monolayer, **c** cells treated with 5 µg/ml doxorubicin and cultured as monolayer, **d** ECM gel-cultured control cells, **e** cells treated with 0.5 µg/ml doxorubicin and cultured in ECM-3-DCC, **f** cells treated with 5 µg/ml doxorubicin and cultured in ECM-3-DCC

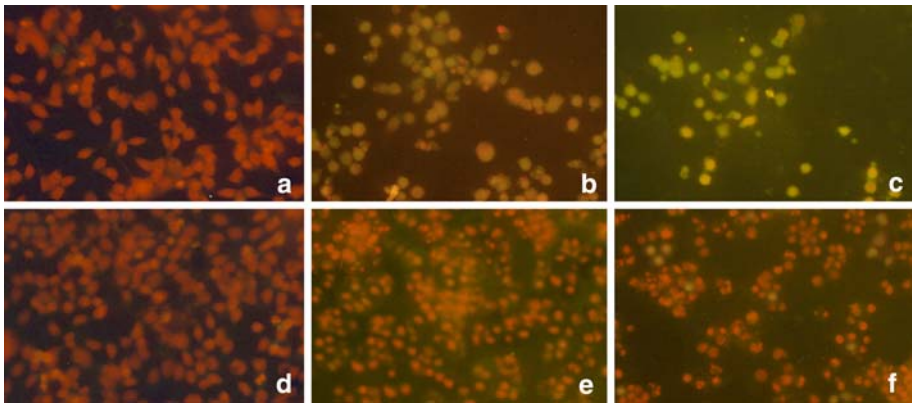

Fig. 3 Effects of ECM gel on the expression of p53 in OSCORT cells. Immunohistochemical detection of p53 in cells cultured for 72 h either on plastic or in ECM gel after treatment with doxorubicin, **a** plastic-cultured control cells, **b** cells treated with 0.5 μg/ml doxorubicin and cultured as monolayer, **c** cells treated with 5 μg/ml doxorubicin and cultured as monolayer, **d** ECM gel-cultured control cells, **e** cells treated with 0.5 μg/ml doxorubicin and cultured in ECM-3-DCC, **f** cells treated with 5 μg/ml doxorubicin and cultured in ECM-3-DCC. p53: *green*, nucleus stained in *red* with propidium iodide. ×200 original magnification

the question has been raised whether the repopulation capacity of these cells has been also protected.

To test the repopulation capacity of OSCORT cultures following treatment with doxorubicin for 4 h, the cells were further incubated in fresh medium with or without ECM gel and cell growth was compared in the monolayer and in the 3-D cultures at the following 24–120 h. At the end of the 4 h treatment with doxorubicin (0.5–10 μg/ml) cell viability showed only 10–23% reduction. These values however showed further reduction during the posttreatment incubation period. For subcultures the number of the plated cells was adjusted on the basis of their trypan-blue exclusion capacity, taken the apoptotic cells into account (Table 1), much less cells were able to proliferate, especially when the cells were transferred from the monolayer cultures.

The repopulating capacity of the cells from the monolayer and from the 3-D cultures was determined 3 days after treatment. The doxorubicin-treated and doxorubicin-untreated OSCORT cells transferred either from monolayer or from ECM-3-DCC were subcultured on plastic surface and their growth rate was investigated. The Fig. 4 shows that untreated OSCORT cells transferred from ECM-3-DCC had a higher growth capacity relative to the cells originated from the monolayer. In fact, the doxorubicin-treated OSCORT cells transferred from the monolayer culture were not able to proliferate at all, although the number of the viable cells plated corresponded to the controls. At the same time, the cytoprotection provided by ECM prevailed upon transferring the cells into plastic dish.

Measurements on the distribution of the cells between the monolayer and ECM gel in 3-DCC gives an estimate for the size of the migrating compartment, in addition the antimigratory and antiproliferative efficacy of a test compound could be determined simultaneously in the same cell culture [6]. Figure 5 showed that OSCORT cells gained protection by ECM to proliferate, however, no reference was given to decide whether cell migration had been equally affected. Therefore in the following experiments, the migrating and the nonmigrating compartments were separated, and the number of cells counted. In OSCORT 3-DCC, 60% of the cells could be recovered in the migrating compartment, however, this value decreased after treatment with a relatively low concentration of doxorubicin. This implies that by as low as 0.01–1.0 μg/ml concentration of doxorubicin migration but not proliferation was affected. It seems that this unique finding is not limited to OSCORT cell cultures, because the ratio of migrating cells to nonmigrating ones changed similarly in other human cell lines treated with doxorubicin and tested for migration in 3-DCC (Fig. 6). It is worthy of note, however, that the differences were less striking than the ones by OSCORT cells. Migration was preferentially affected upon applying the following doxorubicin concentrations: 0.01–1.0 μg/ml in HT-1080 cell cultures, 0.1–1.0 μg/ml in A-431 and in ZR-75.1, 1.0 μg/ml in MDA-MB-231 and in PC-3 cell cultures (Fig. 6).

Discussion

Failure in cancer therapy is largely due to repopulation of the surviving cells after chemotherapy or radiotherapy, for which intrinsic resistance in a certain proportion of the tumor has rightly been made responsible. In this respect, the repopulation is viewed as continuing proliferation of cells with the capacity to regenerate the tumor shrinked after treatment. On the other hand, several lines of studies concluded that microenvironmental factors (poor neovascularization, barrier for drug transport, ECM) could also limit the effectiveness of drug therapy [6, 8, 17, 21, 23, 26].

In the present study the applicability of this latter conclusion for OSCORT cell line was investigated upon comparing its repopulating capacity in monolayer culture and in ECM-based 3-DCC, after doxorubicin

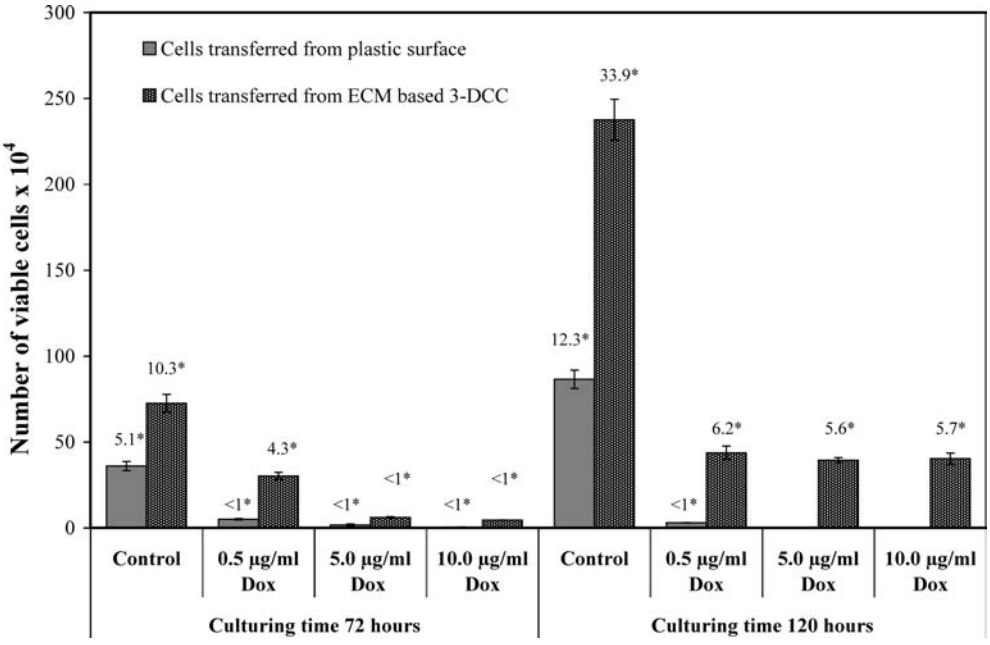

Fig. 4 Repopulation of doxorubicin-treated OSCORT cells in monolayer cultures after cultured either on plastic surface or in ECM-based 3-DCC. OSCORT cells were treated in monolayer culture (i.e., on plastic surface) with doxorubicin for 4 h, and then fresh medium was added with or without ECM gel. Following incubation for 72 h OSCORT cells (as shown on Fig. 5) were removed both from the monolayer (i.e., plastic surface) and from the ECM-based 3-DCC, counted and 7×10^4 viable cells were plated and cultured on plastic surface (i.e., as monolayer culture). After taking into account both trypan-blue exclusion potency and condensed chromatin formation the viability values for the controls, 0.1, 0.5, 5.0, 10 µg/ml doxorubicin-treated cells were as follows: 95, 74, 25, 0 and 0% for cells transferred from monolayer cultures, and 92, 87, 69, 49 and 36% for the cells transferred from the 3-DCC. *To estimate repopulation capacity the number of cells at the indicated time was divided by the number of cells plated and designated as *GRIS* (growth relative to the inoculum size), where value below 1 (<1) shows no growth, i.e., number of cells lower than the inoculum size (7×10^4)

treatment. This experimental setting has created a promising model for studying the relevance of ECM in drug action, because the OSCORT cell line has been established from a chemotherapy-resistant osteosarcoma of a male adolescent, in which certain molecules implicated in invasive growth (β-integrin, MMPs) could be induced by ECM [6, 7]. As Tables 1 and 2 showed, ECM and some of its biopolymers were able to enhance both proliferation and migration of OSCORT cells, and at the same time offer significant protection against the

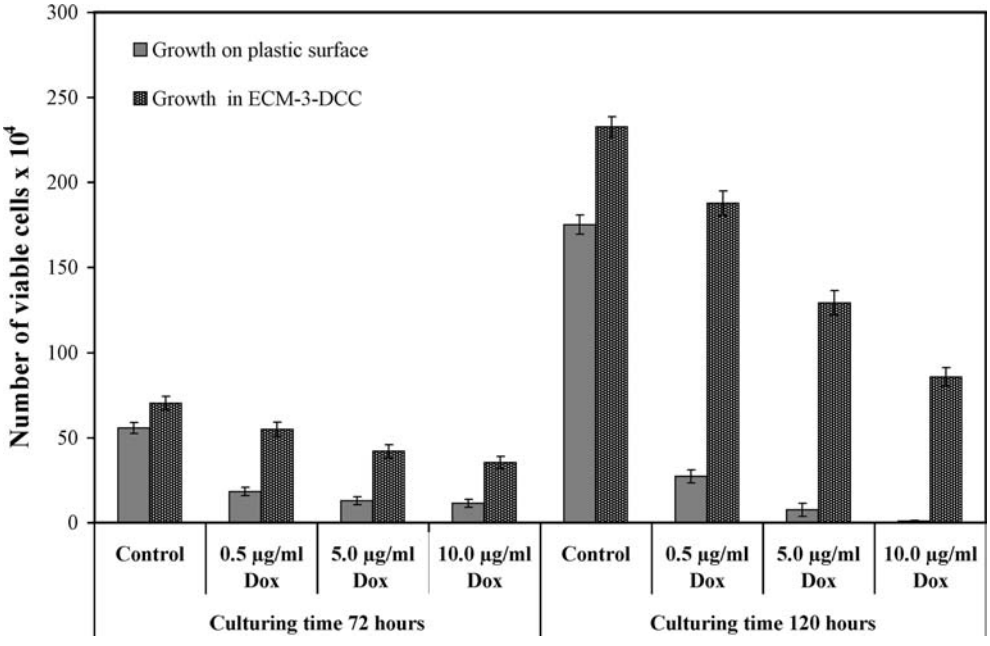

Fig. 5 Growth of doxorubicin-treated OSCORT cell cultures as monolayer culture or in ECM-containing 3-DCC. The effects of 0.1, 0.5, 5 and 10 µg/ml doxorubicin on growth of OSCORT cells cultured on plastic surface or in ECM-3-DCC (ECM gel) were compared using cell counting. The cells were uniformly treated on plastic for 4 h, then they were cultured either on plastic surface or in ECM-3-DCC for the 72 h. In the cell cultures on plastic surface the doxorubicin induced more severe reduction of cell numbers. The *bars* represent the mean ± SD of three independent experiments

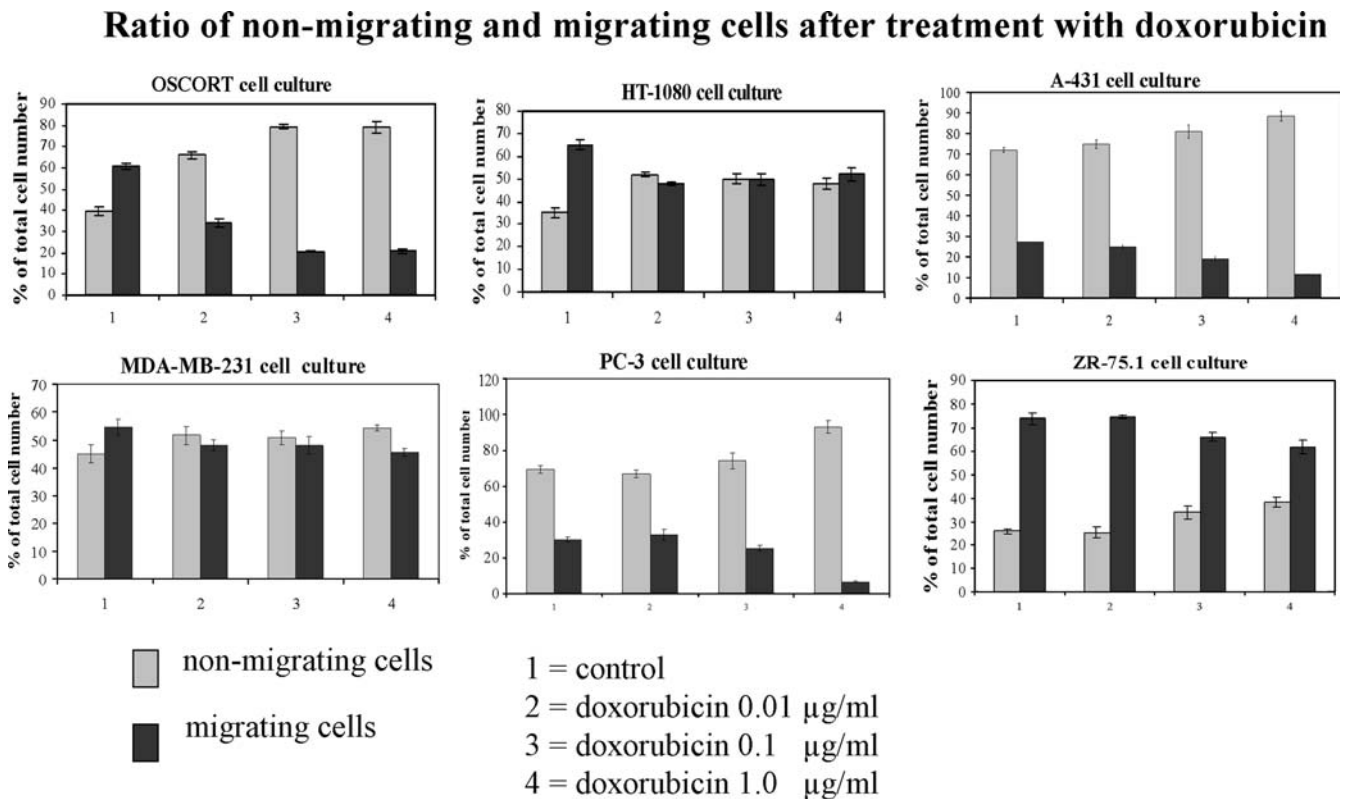

Fig. 6 Effects of ECM gel on the proliferation and migration of different cell cultures after treatment with doxorubicin. Migration was preferentially affected after treatment with a relatively low concentration (0.01–1.0 µg/ml) of doxorubicin (Dox) in different human cell lines (*OSCORT*, *HT-1080*, *A-431*, *MDA-MB-231*, *PC-3*, *ZR-75.1*). Cells tested for migration in 3-DCC

cytotoxic action of doxorubicin. Since doxorubicin treatment in monolayer culture (i.e., without ECM) caused DNA fragmentation at the end of the 4 h treatment, the protection against cytotoxicity could not be explained by the action of ECM on cellular transport and target availability of doxorubicin. It is noteworthy that after removing doxorubicin, DNA damage increased during the following 48 h culture period, but remarkably less extensively in the presence of ECM. In fact, apoptotic index, the parameters of the comet assay and synthesis of p53 were remarkably higher in the monolayer cultures than in the ECM-containing 3-DCC. This implies that after having removed doxorubicin, damage of DNA is further augmented, but controlled by ECM. It is the task of forthcoming studies to decide whether ECM protection is mediated through a more efficient repair mechanism or by inhibiting the cell-death process. This latter possibility may be supported by the much lower expression of p53 in OSCORT cell cultures treated with doxorubicin and postincubated in the ECM-containing 3-DCC. It is possible that the high repopulating capacity of the doxorubicin-treated cells transferred to 3-DCC may have been due to ECM-triggered recovery mechanism, which gain support from our previous study indicating that upon confrontation with ECM both expression and activity of topoisomerase II, which is the major target for doxorubicin, could be elevated [6, 7]. As OSCORT cells were treated with doxorubicin before the exposure to ECM, the increased level of topoisomerase II may have had a possibility to replace the previously damaged enzyme.

The stimulatory action of perlecan on the growth and migration of OSCORT cells is in harmony with the conclusion of other laboratories that heparan sulfate proteoglycans (HSPGs) have multiple roles in tumor invasion [4, 7, 20]. Nevertheless, it has also been reported that HSPGs including perlecan could both promote and reduce carcinoma growth, therefore, its activity is context depending. It is highly probable that, besides the growth factor binding and sequestering capacity of HSPG, the cell-type regulatory mechanisms of tumor cell migration also decides whether HSPG act as promoter or suppressor for invasive growth. In fact, the previously presented experimental setting involving tumor cell migration from the monolayer to the ECM-containing 3-DCC illustrates a clinical situation: when the tumor cells are detached from the primary tumor.

The highly effective protection of OSCORT cells by ECM appeared to be limited to the proliferative capacity of OSCORT cells, because migration was not affected after doxorubicin treatment. Since preferential inhibition of tumor cell migration relative to proliferation was also observed in other human cell lines treated with doxorubicin, the present experimental setting allowed to

illustrate the case when cytotoxic drug-treated tumor cells entering ECM could grow, but with reduced migratory potency.

The surprising differential sensitivity of OSCORT cells against the antimigratory and antiproliferative action of doxorubicin in the presence of ECM support the notion that proliferation and migration represent two contrasting events [24] involving different molecular mechanisms. Consequently, distinct mechanisms may be responsible for the antimigratory and antiproliferative actions of doxorubicin [3].

As a summary of the previously presented study, it has been shown that OSCORT cell growth and its responses to doxorubicin could be remarkably modulated by ECM. Furthermore, ECM-containing 3-DCC as an appropriate model to investigate changes in proliferation and migration of tumor cells has made possible to demonstrate that the repopulating OSCORT cells treated with doxorubicin are less capable to migrate. It is tempting to suggest that understanding the selective modulatory function of ECM on repopulation and migration of tumor cells may set the stage for promising novel pharmacological approaches in cancer therapy.

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