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Resistance of in vivo-selected spontaneously transformed cells and Rous sarcoma virus-transformed cells to macrophage-mediated cytotoxicity

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Abstract. The cytotoxic activity (CTA) of activated peritoneal macrophages (MP) on variant lines of Syrian hamster embryo (HE) cells of differing malignant characteristics was studied. The target cells were a line of low-malignant cells resulting from spontaneous transformation of HE cells in vitro (STHE strain), and malignant variants selected from them in vivo (STHE-LM-4, STHE-LM-8, and STHE-75/18 strains). In addition, we used cells of the HET-SR-1 strain; these are HE cells transformed in vitro by a tumorigenic Rous sarcoma virus (Schmidt-Ruppin strain, RSV-SR), or the TU-SR strain induced by RSV-SR in vivo. Thioglycollate-elicited peritoneal MP from Syrian hamsters were activated in vitro with bacterial levan, LPS or MDP and used as effector cells. MP-mediated cytolysis was determined by means of a 42-h radioactivity release assay with ^{3}H-thymidine-labeled target cells. We found that only the parental STHE cells were susceptible towards fully-activated MP-mediated CTA. All three of the in vivo-selected malignant variants of the STHE cell sublines, as well as the tumorigenic RSV-SR transformants, were resistant to cytolysis by activated MP. Non-activated thioglycollate-elicited MP did not lyse any of the tumor cells studied.
Key words. Natural resistance; tumors; spontaneously- and Rous sarcoma virus-transformed cells; Syrian hamsters; peritoneal exudate cells; macrophages; activation; cytotoxicity.

One of the factors involved in tumor progression is in vivo selection of tumor cells. Effectors of the host's natural resistance (NR) (macrophages, monocytes, natural killer (NK) cells, and neutrophils) may play an essential role in this process [1–3]. It has been shown recently that hamster embryo (HE) cells in vitro, spontaneously transformed during in vivo selection, acquired characteristics of a malignant phenotype. These include tumorigenic activity (TGA) and metastasizing activity (MA), both experimental (EMA) and spontaneous (SMA). Moreover, such in vivo-selected cells variants were resistant to hydrogen peroxide (H_2O_2) damage, and they were also able to release prostaglandin E_2 (PGE_2) when in contact with NK cells [4, 5]. It appears that a number of characteristics of tumor cells (i.e., resistance to H_2O_2 and PGE_2 release, frequently united in clusters), at least partly determine their resistance to NR effector cells. A good correlation of these characteristics with TGA and EMA has been demonstrated [2, 4, 5]. HE cells transformed by Rous sarcoma virus (Schmidt–Ruppin strain) (RSV-SR) in vitro, without any in vivo selection, have also acquired the malignant characteristics, and both the resistance to H_2O_2 damage and the PGE_2-releasing activity [2, 6].

Certain activated cytotoxic macrophages (MP) are well-known effector cells of the host's natural resistance to tumor progression. However, data on their cytotoxic potential for transformed and malignant tumor cells are contradictory [7–9]. Therefore, we examined the susceptibility of two different types of transformed cells, and their in vivo-selected malignant variants, to MP-mediated cytotoxic activity (CTA). Previously, we showed that the low-malignant spontaneously transformed in vitro cells of the STHE strain are a highly susceptible target for MP-mediated CTA [10]. In this study, we compared the

susceptibility of Syrian HE cells, both spontaneously transformed and transformed by RSV-SR in vitro, to MP-mediated CTA. We found that, in contrast to the parental low-malignant STHE HE cells, all the high-malignant Syrian HE cell sublines studied were resistant to cytolysis by activated peritoneal MP.

Materials and methods

Target cells. These were: (1) low-malignant Syrian hamster embryo cells spontaneously transformed in vitro (parental STHE strain); (2) three in vivo-selected malignant STHE cell variants obtained from lung metastases (STHE-LM-4, STHE-LM-8, and STHE-75/18 strains); (3) highly tumorigenic HE cells transformed in vitro by the Schmidt–Ruppin strain of Rous sarcoma virus RSV-SR (HET-SR-1 strain); (4) malignant TU-SR cells obtained from a hamster tumor induced in vivo by RSV-SR. The origin and malignant characteristics of these cells have recently been described in detail[2,4–6]. Monolayer cultures of the target cells were maintained in Eagle's medium with 10% bovine serum and antibiotics by serial in vitro passages, twice weekly.

Effector cells. Peritoneal macrophages (MP) were obtained as previously described[10]. Briefly, adherent peritoneal exudate cells from normal adult Syrian hamsters primed with 5 ml 3% thioglycollate (TG) medium 5 days earlier were collected, pooled, counted, allowed to adhere to plastic, and activated in vitro with different bacterial activators[10]. Differential cell counts were made on Giemsa-Romanovsky stained cells; 65.6 ± 1.8% of the peritoneal exudate cells were macrophages by morphological criteria. After 2 h of incubation of the peritoneal exudate cells in 96-well flat-bottomed plates (Titertek, Flow Labs) at 37 °C with 5% CO_2, non-adherent cells were removed by threefold washing with warm growth medium (RPMI 1640, Flow Labs, Scotland). About 93% of the adherent cells were MP.

Activators. MP-activating agents used were polysaccharide levan produced by *Zymomonas mobilis* (Institute of Microbiology, Latvia), lipopolysaccharide (LPS) from *Escherichia coli*, serotype 026:B6 (Sigma, USA), and N-acetylmuramyl-L-alanyl-d-isoglutamine (muramyldipeptide, MDP) (Behring Diagnostics, USA). The final concentrations of the activators were as follows: levan, 1 mg/ml; LPS, 20 μ/ml; MDP, 10 μ/ml. Previous experiments had shown that these doses were non-toxic, and induced both reactive oxygen intermediates (ROI) which were released upon contact with peritoneal MP[11–13], and MP-mediated cytolysis[10].

Cytolysis assay. The susceptibility of the tumor cells studied to macrophage-mediated CTA was determined by means of a 42-h radioactivity release assay with ^{3}H-thymidine (^{3}H-TdR), as described[10]. Briefly, the subconfluent target cells were prelabeled for 18–20 h with ^{3}H-TdR (Isotop, USSR) (5 Ci/mmol, 1 μCi/ml). The cells were removed by versene-trypsin solution, washed three times, and resuspended in complete medium (RPMI 1640) supplemented with 10% heat-inactivated bovine serum, antibiotics, and HEPES buffer (Flow Labs, Scotland). Then 10^4 target cells were added, together with the activators, to the macrophage monolayers. Tests were performed in triplicate. The effector-to-target ratio was 20:1. The activator agents and target cells were each added in a volume of 100 μl. The plates were incubated for 42 h at 37 °C with 5% CO_2. After incubation 100 μl samples were transferred into scintillation vials, mixed with 10 ml scintillation fluid, and counted on an 'LKB 1219 Racbeta Wallac' counter (Sweden). Results are expressed as the percent of specific ^{3}H release calculated as follows: 100 times ^{3}H cpm released from cells incubated with effector cells, minus ^{3}H cpm released in the presence of medium alone, divided by ^{3}H cpm released in 2% sodium dodecyl sulfate (maximum release), minus ^{3}H cpm released in medium alone. Spontaneous release of ^{3}H by target cells incubated in medium alone ranged from 14.9% to 33.7%.

Statistical analysis was carried out using Students t-test, and p values less than 0.05 were considered to be significant.

Results

Throughout the study, low-malignant HE cells of the parental STHE spontaneously transformed in vitro (never selected in vivo) were used as a positive control. These cells were susceptible to macrophage-mediated cytolysis. The susceptibility towards MP-mediated CTA of three malignant STHE cell variants selected in vivo, as well as RSV-SR tumorigenic transformants, was compared by means of the ^{3}H-TdR assay. Table 1 shows the malignant characteristics of the various cell lines used. The results of the present study are presented in table 2.

The results indicate that the only parental STHE cells show a significant level of cytolysis mediated by TG-elicited peritoneal MP activated with levan, LPS, and MDP ($p < 0.001$).

In contrast, all three spontaneously-transformed malignant STHE cell variants (i.e., STHE-LM-4, STHE-LM-8, and STHE-75/18) were resistant to CTA mediated by activated MP. The same high level of resistance towards MP-mediated cytolysis was observed with the RSV-SR transformed cell lines, HET-SR-1 and TU-SR. Non-activated thioglycollate-elicited peritoneal MP had little or no ability to lyse either the spontaneously-transformed or the RSV-SR- transformed HE cells. The level of cytolysis was always low, and ranged from −2.4 to 5.8% (table 2). These results were quite reproducible. Differences in MP-mediated cytolysis between parental low-malignant STHE HE cells and all the high-malignant cell sublines, studied were highly significant ($p < 0.001$).

The cytolytic activity of activated peritoneal MP was then tested. MP were activated with the following agents of bacterial origin: levan (1 mg/ml), LPS (20 μg/ml) or MDP (10 μg/ml). The MP to target cell ratio was 20:1. The activators were present throughout the 42-h ^{3}H-TdR

Table 1. Malignant characteristics of variant lines of Syrian hamsters embryo cells[2,4–6]

Cell line		Tumorigenic activity	Metastasizing activity		Resistance to H_2O_2	PGE_2 release
Designation	Origin	(log TrD_{50} values)[a]	Experimental	Spontaneous		
STHE (parental)	in vitro spontaneously transformed HE cells (non-selected)	≥2.5	$\leq 10^{-6}$	–	–	–
STHE-LM-4	in vitro spontaneously transformed HE cells (in vivo selected)	≤1.8	$\leq 5 \times 10^{-4}$	–	++	±
STHE-LM-8	in vitro spontaneously transformed HE cells (in vivo selected)	≤1.4	$\leq 5 \times 10^{-3}$	+	+	+++
STHE-75/18	in vitro spontaneously transformed HE cells (in vivo selected)	0.7	$\leq 5 \times 10^{-4}$	+	++	+++
HET-SR-1	HE cells transformed in vitro by RSV-SR (non-selected)	≤1.0	$\geq 10^{-3}$	+++	+++	+++
TU-SR	RSV-SR tumor nodule cells (in vivo induced)	≤0.9	$\geq 10^{-3}$	±	+++	+++

[a] log TrD_{50} = logarithm of 50% transplantation dose.

Table 2. Resistance of in vivo selected spontaneously transformed hamster embryo STHE cell lines to peritoneal macrophage-mediated cytotoxicity: comparison with Rous sarcoma virus (Schmidt–Ruppin strain) transformed cells

Target cells studied	Cell line characteristics		Percent of CTA of the macrophages following treatment with			
	Transforming agent	Selection	Levan	LPS	MDP	Medium
STHE (parental)	spontaneous	none	16.6 ± 2.5*	18.7 ± 0.3*	22.4 ± 0.6*	4.1 ± 0.1 (10)
STHE-LM-4	spontaneous	in vivo	1.6 ± 2.9	1.5 ± 1.2	3.2 ± 0.1	5.8 ± 3.6 (3)
STHE-LM-8	spontaneous	in vivo	6.2 ± 0.9	6.7 ± 2.6	6.6 ± 4.9	4.2 ± 4.3 (3)
STHE-75/18	spontaneous	in vivo	1.6 ± 0.9	−4.5 ± 5.5	−1.1 ± 1.2	−2.4 ± 4.2 (3)
HET-SR-1	RSV-SR	none	4.4 ± 0.1	0.8 ± 0.6	3.3 ± 1.5	−0.3 ± 0.6 (4)
TU-SR	RSV-SR	in vivo[a]	1.1 ± 1.0	2.6 ± 0.6	4.4 ± 4.6	1.3 ± 0.6 (6)

Specific CTA was tested 42 h after incubation of the ^{3}H-thymidine-labeled target cells with thioglycollate-elicited peritoneal MP. Summarized data (mean ± standard error) of three or more independent experiments are presented (*$p < 0.001$). The number of separate experiments is given in parentheses. [a] RSV-SR in vivo-induced tumor cells.

assay. The activated MP showed an enhanced cytolytic activity against the low-malignant parental STHE HE cells ($p < 0.001$). There was no significant enhancement of cytolytic activity against any of the five high-malignant strains tested (table 2). No significant difference in the MP-activating potential of the different bacterial agents was observed.

Discussion

The parental STHE strain HE cells, spontaneously transformed in vitro and never selected in vivo, were previously shown to be characterized by (1) a relatively low level of TGA; (2) extremely low metastasizing activity, both experimental (EMA) and spontaneous (SMA); and (3) the absence of detectable tumor specific transplantation antigen, TSTA[2,4,5]. In contrast, all the STHE cell variants selected in vivo which were studied, and also the cells transformed by Rous sarcoma virus in vitro (or induced in vivo) showed a malignant phenotype[2,4–6]. These characteristics are summarized in table 1. The EMA of parental STHE cells was rather low: only 1 out of 10^6 cells inoculated intravenously was able to form pulmonary metastases. In contrast, the EMA of the sublines selected from these cells in vivo (STHE-LM-4, STHE-LM-8 and STHE-75/18) was 100- to 1000-fold higher[4,5]. RSV-SR transformants studied (HET-SR-1 and TU-SR lines) showed extremely high levels of tumorigenic activity (the logarithm of the 50% transplantation dose, TrD_{50}, was near or below 10 cells), and of EMA, and even of SMA in the case of the HET-SR-1 cell line[2,6]. All malignant HE cell lines studied had high levels of both H_2O_2-resistance and PGE_2-releasing ability, while the parental STHE cells were negative for both these characteristics[2,4–6].

Recently, in our laboratory, it has been shown that original low-malignant STHE cells are highly susceptible targets for cytolytic and cytostatic activity mediated by macrophages[10,14], NK cells[15], and polymorphonuclear neutrophils[16,17]. Tumor cells are heterogeneous in their susceptibility to macrophage-mediated CTA. In some tu-

mor systems in rodents, a decrease of susceptibility of metastatic tumor variants to certain forms of activated MP cytolysis has been shown [8, 9, 18–26].

Our previous studies showed that parental STHE cells are highly susceptible to CTA of peritoneal MP when these are activated either with bacterial products (such as levan, LPS, MDP, and proper-myl) or chemically (for example by phorbol 12-myristate 13-acetate, PMA; dihydropyridine derivates, PP-256). However, resident and non-activated TG-elicited peritoneal MP failed to produce significant CTA against the STHE cells. The susceptibility of the STHE cells to MP-mediated cytolysis was, however, potentiated by short-term pretreatment with actinomycin D [10].

In this study, we compared the susceptibility of low- and high-malignant HE cells towards cytolysis by hamster peritoneal macrophages activated in vitro with three immunomodulators of bacterial origin: levan, LPS and MDP. These activators increased the susceptibility of the low-malignant parental strain of STHE HE, but did not affect the susceptibility of the five high-malignant strains tested (table 2).

We thus demonstrated that the three STHE sublines selected in vivo, and the two tumorigenic lines produced by RSV-SR transformation, had lost their susceptibility to CTA mediated by peritoneal macrophages. The cells which showed resistance to cytolysis were those which had a high tumorigenic and metastasizing activity, and also showed other features typical of malignant cells, such as a high level of H_2O_2 resistance and of PGE_2-release. Interestingly, the cell line HET-SR-1 (produced by RSV-SR transformation in vitro) showed a high level of resistance to cytolysis although it had never been selected in vivo.

Our findings were consistent with many previous observations [8, 9, 18–24]. However, Fidler [7] found no differences in susceptibility to MP-mediated CTA between low- and high-metastatic variants of B16 melanoma.

One of the proposed mechanisms for tumor heterogeneity and tumor progression is macrophage-mediated selection both in vivo and in vitro [23, 24]. Recently, we showed that in vitro co-cultivation of the parental STHE HE cells with LPS-activated peritoneal macrophages results in the selection a number of STHE cell variants which are resistant both to activated MP and to different concentrations of exogenous H_2O_2 [27, 28]. Moreover, STHE cell variants selected in this way simultaneously acquired characteristics of a malignant phenotype, i.e. they showed increased tumorigenic and metastatic activity in comparison to the parental STHE cells [28, 29]. The exact mechanism(s) of the resistance of transformed malignant HE cells to cytolysis mediated by Syrian hamster peritoneal macrophages remains to be elucidated. Certain activated MP are known to be sources of interleukin-1, tumor necrosis factor (TNF), reactive oxygen intermediates (mainly, H_2O_2), arachidonic acid metabolites, and many other cytotoxic products (for review, see ref. 1–3, 30). Selective resistance to TNF and H_2O_2 appear to be one of the mechanisms for resistance of some malignant cell variants towards activated macrophages [23, 24, 27, 28, 31, 32].

Taken together, these data indicate that activated macrophages play a critical role, both in vivo and in vitro, in the selection of transformed cells of varying levels of malignancy.

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