TUMORIGENICITY AND METASTASIZATION OF STHE CELLS, WITH A LOW LEVEL OF MALIGNANCY, DURING IN VITRO SELECTION BY PERITONEAL EXUDATE CELLS

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In the modern view one factor in tumor progression is selection of malignant forms of cells in vivo, in which an important role is played by effector cells of the system of natural resistance (NR), namely macrophages (Mph) and monocytes, natural killer cells, neutrophils and, possibly, other cells [3]. In the course of selection, cell forms surviving in vivo differ from the parental cells in their more marked malignant properties — their tumorigenic (TGA) and metastatic (MA) activity, and also in their higher level of resistance to effectors of NR [3, 4]. Many primary tumors, like cell lines obtained from them in culture, are heterogeneous and consist of phenotypically different cell subpopulations [3, 9, 15]. One of the most important phenotypic features, serving to distinguish the subpopulations of tumors cells, is ability to metastasize.

It was shown previously that during in vivo selection of spontaneously transformed cells of the STHE strain, with a low level of malignancy, the surviving forms of cells were characterized by more marked malignant properties (TGA, MA), but at the same time, unlike the parental cells, they were resistant to hydrogen peroxide (H_2O_2) and they secreted an increased amount of prostaglandins E (PGE) on contact with natural killer (NK) cells [4-6]. Correlation was observed between these two discrete characteristics of the tumor cells (resistance to H_2O_2 and secretion of PGE) and TGA and experimental MA [4-6].

In previous studies the writer showed that cells of the STHE strain selected in vivo were resistant to the cytotoxic action (CTA) of activated Mph [1]. Different forms of STHE which we obtained during in vitro selection by cocultivation of parental STHE cells with peritoneal exudate cells (PEC), activated by lipopolysaccharide (LPC), most of the adherent cells of which were Mph (unlike the original STHE cells or versions of STHE selected in vitro with resident PEC), were resistant to CTA of activated Mph and to H_2O_2 in radioisotope tests with ^{3}H-thymidine [2].

The aim of this investigation was to compare TGA and MA (spontaneous and experimental – SMA and EMA respectively) forms of STHE, obtained by selection in vitro with resident and activated PEC.

EXPERIMENTAL METHOD

Experiments were carried out on intact male Syrian hamsters aged 4-6 months, bred at the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR. Cells of a strain of Syrian hamster embryonic fibroblasts, spontaneously transformed in vitro, and with a low level of malignancy [6], and alternative forms of strain STHE obtained by 10 consecutive cycles of in vitro selection by cocultivation of the original parental STHE cells with PEC from intact Syrian hamsters, resident and activated by LPS from *E. coli* ("Sigma," USA) (PEC_r and PEC_a respectively), were used as the target cells. The PEC contained 1.9 ± 0.8% neutrophils, 56.4 ± 3.3% Mph, and 41.6 ± 3.5% of lymphocytes [2]. The target cells were maintained in culture on Eagle's medium with lactalbumin hydrolysate, containing 10% bovine serum and gentamicin, and subcultured twice a week.

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TABLE 1. TGA of Forms of STHE Cells Selected in Vitro with PEC_r and PEC_a, at Different Stages of Selection (pooled data)[a]

PEC used for selection in vitro	Number of cycles of selection	Results of transplantation test after inoculation: Dose of STHE cells $(1,2-1,6)\cdot10^1$	$(1,2-1,6)\cdot10^2$	$(1,2-1,6)\cdot10^3$	$(1,2-1,6)\cdot10^4$	log TD^b_{50} (M ± m)
PEC_r	4	0/24	7/24	19/24	24/24	2,65±0,01**
	6	4/27	8/27	21/27	27/27	2,45±0,25*
	10	1/31	8/31	24/31	31/31	2,58±0,06**
PEC_a	1	5/18	5/18	15/18	18/18	2,20±0,29
	2	1/18	4/18	17/18	18/18	2,43±0,29
	4	8/26	7/26	21/26	26/ 26	1,80±0,22*
	5	7/26	8/26	23/26	26/26	2,20±0,22*
	10	9/25	12/25	22/25	25/25	1,67±0,11***
Control—intact STHE cells	—	0/65	2/65	30/65	65/65	3,17±0,07

Legend. [a]Pooled data of 3-5 experiments, control – 11 experiments. [b]Logarithm of dose of tumor cells giving growth of subcutaneous tumors in 50% of animals inoculated [16]. [c]Parameters of successful transplantation of test cells: numerator – number of hamsters with growing tumor, denominator — number of animals inoculated. Here and in Tables 2 and 3, statistically significant differences compared with control values: *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$.

TABLE 2. SMA of Forms of STHE Cells, Selected in Vitro with PEC_r and PEC_a, at Different Stages of Selection (pooled data)[a]

PEC used for selection in vitro	Number of cycles	Number of SM in lungs after transplantation of test STHE cells[b]: frequency of cases[c]	mean number (M ± m)	limits of variations
PEC_r	4	22/24*	14,6±2,5*	0—46
	6	21/24**	9,6±1,3*	0—24
	10	27/29***	13,0±2,0*	0—44
PEC_a	1	17/18**	20,7±7,1*	0—106
	2	16/18*	13,7±3,1*	0—47
	4	24/25***	25,2±3,9***	0—76
	5	23/24***	19,2±5,0*	0—94
	10	22/25***	18,4±2,5***	0—48
Control—intact STHE cells	—	38/65	4,4±0,9	0—36

Legend. [a]Pooled results of 3-5 experiments, control – 11 experiments (see Table 1). [b]Autopsy of animals on 60th day after subcutaneous inoculation. [c]Numerator, number of hamsters with SM; denominator, number of animals inoculated.

TGA of the test STHE forms was determined in a quantitative transplantation test in vivo. For this purpose four different doses of cells of each form of STHE, differing by a factor of 10, were injected subcutaneously into normal hamsters (groups of 5-7 animals). These doses as a rule ranged from $(1.2\text{-}1.6)\cdot10^1$ and $(1.2\text{-}1.6)\cdot10^4$ cells respectively, and each dose was given in a volume of 0.2 ml. After 2 months the animals were anesthetized and the value of log 50% of the transplanted dose (log TD_{50}) for each form of STHE was determined [16]. Next, the size of each subcutaneous tumor growing in each hamster was measured in square centimeters on the 60th day after transplantation of the test forms of STHE cells (or sooner, should the animal die), by the formula: size of tumor = $\pi ab/4$, where a denotes the maximal diameter of the tumor, b the minimal diameter of the tumor in the direction perpendicular to a [8]. The TGA of each form of STHE was tested in three or more experiments.

SMA was determined 2 months later at autopsy on the animals which had received four subcutaneous injections of doses of cells of the test forms of STHE, differing by a factor of 10 (see above: determination of TGA). As a rule the spontaneous metastases (SM) of the test cells were located in the lungs. The number of lung metastases in each animal was counted under a magnifying glass with a magnification of 25-30 times, the five separate lobes of the lungs being placed between two flat surfaces of glass Petri dishes. For each cell form the average number of lung metastases was calculated. The appearance of 20 or more metastatic nodes in the lungs was regarded as an indicator of SMA of that particular cell strain [5].

TABLE 3. EMA of Forms of STHE Cells Selected in Vitro with PEC_r and PEC_a, at Different Stages of Selection (pooled data)[a]

PEC used for selection in vitro	Number of cycles of selection	Number of experimental metastases in lungs after inoculation of STHE cells[b] dose of STHE cells					
		$(1,8—2,3)\cdot10^5$			$(1,8—2,3)\cdot10^6$		
		frequency of cases[c]	mean number (M ± m)	limits of variations	frequency of cases[c]	mean number (M ± m)	limits of variations
PEC_r	4	9/20**	1,0±0,4*	(0—6)	20/20***	11,0±1,5***	(2—26)
	6	15/17***	5,0±1,3**	(0—19)	21/21***	45,1±7,8***	(2—134)
	10	10/18**	2,3±0,8*	(0—12)	18/18***	53,8±9,2***	(1—139)
PEC_a	1	17/18***	10,1±0,2**	(0—43)	17/17***	55,1±8,0***	(18—133)
	2	10/12***	5,6±1,1***	(0—12)	12/12***	42,5±6,4***	(17—86)
	4	20/20***	28,4±10,3*	(5—173)	19/19***	135,6±32,2***	(34—558)
	5	18/18***	30,1±4,6***	(3—72)	18/18***	275,4±29,5***	(58—500)
	10	17/17***	17,5±3,2***	(3—49)	17/17***	183,7±30,7***	(47—500)
Control—intact STHE cells	—	0/45	0	—	26/51−	2,5±0,4−	(0—14)

Legend. [a]Pooled results of 2-3 experiments, control – eight experiments. [b]Autopsy on animals on 22nd-24th day after intravenous injection. [c]Numerator – number of hamsters with EM; denominator – number of animals inoculated.

EMA was determined by the method described previously [6]. Single-cell suspensions of the test forms of STHE were obtained by removing them from the glass with versene solution, after which they were resuspended in culture medium and, under ether anesthesia, injected into normal hamsters (groups of 6-8 animals), in two different doses of cells differing by a factor of 10 [usually $(1.8\text{-}2.3)\cdot10^5$ and $(1.8\text{-}2.3)\cdot10^6$ cells respectively], into the retro-orbital venous sinus of each animal in a volume of 1 ml. As a rule all the inoculated animals were anesthetized and autopsied at the same time, 22-24 days after inoculation of the test STHE cells (or sooner should the animals die), after which the distribution and number of visible metastases in the lungs or other organs of the animal were determined. The average number of lung metastases was calculated for each form of cell, and for each dose of cells. The minimal dose of cells causing the formation on average of about 10 experimental metastases (EM) in the lungs was regarded as the presence of a positive EMA. Each form of STHE cells was tested in two or three experiments.

The numerical results were subjected to statistical analysis by the chi-square test and by Student's t test.

EXPERIMENTAL RESULTS

The TGA, SMA, and EMA of three forms of STHE cells, selected in vitro after 4, 6, and 10 cycles of cocultivation with resident PEC_r, and five forms of STHE selected in vitro after 1, 2, 4, 5, and 10 cycles of cocultivation with PEC_a, and of the original parental STHE cells, not subjected to cocultivation with PEC, and which served as the principal control, were investigated.

Investigation of TGA of forms of STHE selected in vitro. Pooled results of the study of TGA of types of STHE selected in vitro with PEC_r and PEC_a, at different stages of selection, are shown in Table 1. Some decrease was observed in log TD_{50} at all stages of selection of the STHE cells in vitro with PEC_r and PEC_a; a significant decrease in TD_{50} (by 1.5log), evidence of intensification of TGA, was found, moreover, in the later stage of selection – after 10 cycles of cocultivation of STHE cells with PECa ($p < 0.001$). Inoculation with the minimal dose (10 cells) of the test forms of STHE caused growth of subcutaneous tumors in about 30% of animals inoculated with the forms of STHE obtained after 1, 4, 5, and 10 cycles of selection with PEC_a, and in 15% of hamsters (four of 27), inoculated with forms of STHE obtained after six cycles of selection with PEC_r, whereas on transplantation of the same number of cells of the parental strain of STHE, subcutaneous growth of the cells could not be detected (Table 1). It was also observed that, unlike the original parental STHE cells, all forms of STHE tested that had been selected in vitro with PEC_r and PEC_a grew more quickly and gave growth of significantly larger subcutaneous tumor nodes (data not given), although differences in the size of the subcutaneous nodes between those selected in vitro at different stages of selection with PEC_r and PEC_a were found to be not statistically significant ($p > 0.05$).

The study of SMA of forms of STHE selected in vitro. Table 2 gives the pooled results of determination of the frequency and number of SM found at autopsy on animals inoculated subcutaneously 2 months previously with cells of the test forms of STHE (see the results of investigation of TGA above). As will be clear from the data in Table 2, SM of forms of STHE were located mainly in the lungs in 87-96% of the inoculated animals. Analysis of data obtained by counting SM in the lungs showed that forms of STHE selected in vitro with PEC_r at different stages of selection gave growth of metastases in 87-93% of inoculated animals. The average number of SM in these groups was 2-2.7 times greater than the control values ($p < 0.05$). Meanwhile, forms of STHE cells obtained by selection in vitro with PEC_a caused SM in the lungs in 88-96% of experimental animals independently of the number of cycles of selection of STHE cells (beginning with the first). The average number of metastases in the animals of these groups was significantly greater than the number of SM in the control animals, but as a rule it did not exceed 20-25. Comparison of the number of SM within the experimental groups of animals revealed no significant differences depending on the number of cycles of selection ($p > 0.05$).

By contrast with these data, as a rule solitary SM in the lungs were found in only 38 (58.5%) of the 65 control animals inoculated with parental STHE cells. The mean number of SM in the lungs of the control animals was significantly lower than that in animals of the experimental groups, inoculated with types of STHE cells selected in vitro with PEC_r and PEC_a (Fig. 2).

Investigation of EMA of forms of STHE selected in vitro. The pooled data of determination of EMA at autopsy on the animals 22-24 days after intravenous injection of two doses of cells of the test forms of STHE, differing by a factor of 10, are shown in Table 3. They demonstrate that injection of the minimal dose (about $2 \cdot 10^5$ parental STHE cells) did not lead to the formation of EM in the lungs in any of the 45 animals studied in the control groups, whereas in animals of the experimental groups, inoculated with forms of STHE cells selected in vitro with PEC_r the experiments revealed in 45-88% of cases growth of a small number (on average from one to five) of EM in the lungs.

Conversely, when hamsters were inoculated with the same dose of cells (about $2 \cdot 10^5$) of forms of STHE selected in vitro with PEC_a, a marked statistically significant increase in the intensity of EM formation was observed in 83-100% of the animals after the first cycle of selection, and the increase was greater after the fourth, fifth, and 10th cycles of selection; with an increase in the number of cycles of selection, moreover, the number of metastatic nodes in the lungs increased significantly (Table 3).

An increase in the dose of inoculated cells to about $2 \cdot 10^6$/ml led to the formation of solitary EM in half (26 of 51) of the cases in the control groups with parental STHE cells. Meanwhile, in 100% of animals inoculated with forms of STHE cells selected in vitro with PEC_r and PEC_a, EM appeared, and in the late stages of selection with PEC_a (after four, five, and 10 cycles), the presence of large and confluent metastases in the lungs was noted.

Investigation of EMA of forms of STHE selected in vitro thus evidently suggests that as a result of selection in vitro with LPS-activated PEC, forms of STHE selected are characterized by a stronger malignant potential, whose level rose in the late stages of selection: a tenfold increase in EMA was noted (the minimal dose was about $2 \cdot 10^5$ cells); inoculation with a larger dose (about $2 \cdot 10^6$) of test cells led to a significant increase in the number of EM. It must be emphasized that it is these forms of STHE cells, selected with PEC_a (as we showed in a previous study [2]), that were resistant at all stages of selection to the CTA of activated peritoneal macrophages and H_2O_2 in vitro.

Forms of STHE selected in vitro with PEC_r were less able to form EM in the lungs (the minimal dose was about $2 \cdot 10^6$ cells), but like forms of STHE selected in vitro with PEC_a, they differed significantly according to this characteristic from the original parental STHE cells, the minimal metastasis-forming dose of which was over $2 \cdot 10^6$ cells. It is worth mentioning here that one form of STHE obtained after 10 cycles of selection in vitro with PEC_r, giving growth of EM in the lungs in 61% of cases after injection in a dose of $2 \cdot 10^6$ cells, also proved resistant in some experiments to the CTA of H_2O_2 [2].

The investigations thus showed that in the course of selection in vitro with PEC_a, forms of STHE cells with a low degree of malignancy (unlike parental cells and forms selected with PEC_r), are more resistant to the CTA of Mph and H_2O_2 in vitro [2], and at the same time, they are characterized by more malignant properties (TGA, MA) in vivo. In our view, during selection in vitro of forms of STHE resistant to the CTA of Mph and H_2O_2, with signs of a malignant phenotype, the principal selection factor of PEC in our system was evidently Mph and also, perhaps, neutrophils, for we know that it is these effector cells of NR that produce H_2O_2 and other active forms of oxygen during the process of activation. There is some evidence that Mph from PEC have the property of potentiating TGA in a number of experimental systems [7, 10, 17]. Moreover, many investigators have found heterogeneity in sensitivity of cell lines with high and low degrees of malignancy to the direct CTA of Mph [11-13, 17-20].

The most clearly defined trait of the malignant phenotype of forms of STHE (resistant to H_2O_2) which we selected in vitro from PEC_a proved to be their ability to form EM in the lungs after intravenous injection into animals, which was more marked in the late stages of selection (Table 3). Although during selection in vitro of PEC_a forms of STHE became resistant to H_2O_2, this is evidently insufficient for them to exhibit ability to undergo spontaneous metastasization; a similar conclusion was expressed previously with respect to STHE cells during selection in vivo of their H_2O_2-resistant forms [6]. SMA was discovered chiefly in the lungs of the experimental animals, and the degree of its expression during selection of forms of STHE in vitro with PEC_a was at a relatively low level (on average about 20 nodes in the lungs) (Table 2). Under these circumstances forms of STHE obtained by selection with PEC_a showed an increase of 7.5-15 times in TGA compared with parental STHE cells, whereas forms of STHE selected in vitro with potentiated PEC_r TGA by a lesser degree (Table 1).

The change in phenotype of the tumor cells during selection in vivo and in vitro, which we found in many tumor systems (including in our own), may perhaps apply, not only to the features we studied, but also to other characteristics essential for intercellular interaction. Intercellular communications may evidently play an important role here, by stabilizing the cell population and restricting the degree of cellular diversity [14]. In this connection it would be interesting to discover whether forms of tumor cells differing in tumorigenicity and metastatic potential, possess an altered character of intercellular junctions. We are currently studying changes in the character of intercellular junctions in a population of STHE cells selected in vitro with the aid of resident and activated macrophages.

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