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Human tumor cells, modified by a novel pressure/crosslinking methodology, promote autologous lymphocyte proliferation and modulate cytokine secretion

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Abstract Hydrostatic pressure (P) combined with membrane protein crosslinking (CL) by adenosine dialdehyde (AdA) can render tumor cells immunogenic. We have recently shown that PCL treatment of murine tumor cells augmented the presentation of MHC-restricted tumor-associated antigens and enhanced cell-mediated immunity. In cancer patients inoculated with autologous PCL-modified tumor cells, a significant delayed-type hypersensitivity response was elicited. Since the balance between cell-mediated immunity and humoral immunity is reciprocally controlled by immunoregulatory cytokines, we have examined the proliferative response and cytokine secretion pattern in cultures of human peripheral blood mononuclear cells (PBMC) stimulated by autologous PCL-modified and unmodified tumor cells. These tumor cells were obtained from freshly resected tumor tissue of 16 patients with colon (8), lung (4) and renal (4) carcinomas. The results demonstrated that PCL-modified tumor cells promoted an increase in PBMC proliferation in 5 out of 8 (63%), 1 out of 4 (25%) and 4 out of 4 (100%) colon, lung and renal cell carcinomas. Fourteen of the above cultures were also analyzed for the secretion of interleukin-10 and interferon-γ. Overall, a substantial decrease in IL-10 secretion was detected in 9 out of 14 (64%) cultures while a reciprocal increase in interferon-γ secretion was noted in 8 out of 14 (57%) cultures. Our results confirmed that PCL-modified human tumor cells of different etiologies can modulate the pattern of cytokines released from stimulated autologous lymphocytes. Such a procedure could prove valuable in the production of autologous tumor vaccines.

Key words Hydrostatic pressure · Crosslinking · Cytokines · Immunotherapy · Tumor vaccine

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Introduction

There is mounting evidence that tumors can induce local immunosuppression of T-helper cells (Th) by modulating the secretion of cytokines within the tumor milieu [23]. This may be due to constitutive expression of cytokines by the tumors [3] or to the tumor's ability to modulate the response of Th cells through other mechanisms [11, 29]. With respect to the former, a number of animal and human tumors have been shown to secrete T-helper type 2 (Th2) cytokines, such as interleukin-4 (IL-4) and IL-10, which are known to suppress T-helper type 1 (Th1) cellular differentiation [16, 33, 19] hence impeding the secretion of Th1 cytokines which are necessary for the expansion of cytotoxic T cells (CTL).

We have previously shown that murine tumor cells exposed to a short period of hydrostatic pressure (P) in the presence of the crosslinker (CL) adenosine dialdehyde become potent immunogens with enhanced presentation of putative MHC-restricted tumor-associated antigens [21]. Mice immunized with PCL-modified irradiated syngeneic tumor cells have been shown to resist a lethal challenge of viable tumor cells [6]. Splenocytes obtained from such immunized animals displayed tumor-specific cytotoxicity in vitro while skin testing provoked tumor-specific delayed-type hypersensitivity (DTH) in vivo [21]. In view of these findings, the protective immunity afforded by PCL-modified tumor cells seems to operate via a cell-mediated mechanism.

In the course of a recently concluded phase I clinical study, the majority of unimmunized cancer patients were found to mount a significant DTH response against autologous PCL-treated, irradiated tumor cells but not against unmodified, irradiated tumor cells (Dr. H. Brenner, Sheba Medical Center). Since the balance between cell-mediated immunity and humoral immunity is controlled by the balance between immunoregulatory cytokines [9, 2], we postulated that PCL-modified tumor cells enhanced the cell-mediated immunity by modulating the cytokine profile in responding lymphocytes. In the present study we examined this postulate by analyzing the proliferative responses and cytokine secretion patterns of autologous peripheral blood mononuclear cells (PBMC) stimulated in vitro by PCL-modified or unmodified tumor cells. Our results indicated a specific bias in lymphocyte response against the PCL-modified cells favoring a cell-mediated-immunity-activating pattern. This observation, together with the PCL-enhanced antigenic presentation of putative tumor-associated antigens, endows the PCL-based tumor vaccine with attractive properties for application as a cancer immunotherapy vehicle.

Patients and methods

Patients

Sixteen cancer patients, 10 male and 6 female aged 49–80 years, were randomly selected from patients diagnosed as having one of three types of cancer: colon carcinoma, lung carcinoma and renal-cell carcinoma (RCC) (Table 1). These patients had not received chemotherapy within 4 weeks or immunotherapy within 6 weeks of the entry date into the study. All participants provided informed consent before entering the trial, which was approved by the internal review boards of the participating institutions.

Preparation of tumor cells

Single-cell suspensions of the tumors were prepared as described [31]. In the majority of cases, resected tumors were processed within 1 h while some were processed for up to 3 h after surgery; during this interim period, the tumors were kept in cold saline. In the first stage, necrotic and fatty tissues were removed and the tumor was dissected into small fragments (1–3 mm^3). These fragments underwent enzymatic digestion for 2–3 h in a mixture containing hyaluronidase (Sigma H-6254, 2000 units/ml) collagenase (Sigma C-5138, 5000 units/ml) and DNase (Sigma D-5025, 300 units/ml) in RPMI-1640 medium. The tumor cell suspension was washed once in Hanks' balanced salt solution (HBSS), separated on a Ficoll Paque cell density gradient (catalog no. 17-0840-02; Pharmacia Biotech, Sweden) and washed twice in HBSS. Cell viability was assessed by trypan blue exclusion. Cells were then PCL-treated as outlined previously [8]. Briefly, up to 5×10^7 tumor cells were suspended in HBSS containing 10 mM adenosine dialdehyde and exposed to 1200 atmospheres (122 MPa) of hydrostatic pressure produced by a pressure bomb of 40 ml capacity (Aminco, American Instrument Co., Md.). Cells were allowed to complete 1 h of incubation in adenosine dialdehyde, washed three times in HBSS and irradiated at 100 Gy. PCL-processed cells were cryopreserved in complete medium [composed of RPMI-1640 medium containing 10% fetal calf serum (catalog no. 04-001-1A; Biological Industries, Beit Haemek, Israel), 2 mM L-glutamine (catalog no. 03-020-1C, Biological Industries), 1 mM sodium pyruvate (catalog no. 03-042-1B, Biological Industries), non-essential amino acids (catalog no. 01-340-1B, Biological Industries), and antibiotics (catalog no. 03-031-1B, Biological Industries)] + 10% dimethylsulfoxide at –70 °C until use. These cells served as stimulators in the in vitro sensitization (IVS) cultures (see below). Unmodified tumor cells were stored under the same conditions and served as the control stimulators in the IVS assays.

IVS culture

Ten days after surgery, 30 ml heparinized blood was drawn and diluted 1:2 in HBSS. PBMC were harvested from the interphase on a Ficoll gradient as described [7], washed twice in HBSS and kept at 4 °C until use. These served as the responders in the IVS cultures, which were set up as follows. Triplicate sets of 2×10^5 freshly prepared PBMC were incubated at 37 °C for 5 days with 5×10^4–4×10^5 sensitizing cells (PCL-modified or unmodified tumor cells) in 200 µl culture medium, in a 96-well microtiter plate (Nunc, Roskilde, Denmark). As positive controls, PBMC were incubated with 0.9 µg/ml (final concentration) of phytohemagglutinin (catalog no. HA15 reagent grade; Murex Diagnostics Ltd., UK) and 1:20 (final dilution) anti-CD3, prepared from culture medium of the 454 hybridoma (Kindly provided by J. Lawrence, Cornell University, NY). On day 5 of IVS culture, 150 µl from each well was removed for cytokine measurements and replaced by 150 µl fresh medium. For the last 4 h of the assay, the cultures were pulsed with [^{3}H]thymidine (1 µCi/well of 50 Ci/mmol, catalog no. 24042; ICN Pharmaceuticals, Costa Mesa, Calif.) and subsequently harvested (Filtermate Cell Harvester 196, Packard). The extent of [^{3}H]thymidine uptake, reflecting cell sensitization and proliferation, was measured as cpm with a liquid scintillation analyzer (Tri-carb 2100 TR liquid scintillation counter, Packard).

Cytokine determination

Cytokine measurements were performed in antibody-coated 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) using the enzyme-linked immunosorbent assay method. For IL-10 determination, polyclonal rabbit anti-(human IL-10) (catalog no. 80-3717-01; Genzyme, Cambridge, Mass.), biotinylated rabbit anti-(human IL-10) (catalog no. 18562D; PharMingen, San Diego, Calif.) and streptavidin-conjugated horseradish peroxidase (HRP) (catalog no. 016-030-084; Jackson ImmunoResearch Lab, West Grove, Pa.) were employed. For IFNγ determination, mouse anti-(human IFNγ) (catalog no. 1598-00, Genzyme), rabbit anti-(human IFNγ) (catalog no. IP-500, Genzyme) and goat anti-(rabbit HRP) (catalog no. 111-036-003, Jackson ImmunoResearch Lab) were used. Recombinant IFNγ (catalog no. 80-3348-01, Genzyme) and IL-10 (catalog no. 2226-01, Genzyme) were used to establish a standard curve for quantifying the cytokine concentrations (pg/ml) in each sample. The specific activity of the IFNγ was at least 1×10^7 U/mg, where one unit (U) is defined as the amount of IFNγ required to provide 50% inhibition of viral replication. These units are equivalent to the NIH human IFNγ reference standard.

Presentation of proliferation index and cytokine secretion

Proliferative response in IVS

The cell proliferation index of PCL-modified tumor cells, I_{prolif}, was calculated as follows:

$$I_{prolif} = (A - B) / (C - D)$$

where A (cpm): responders (PBMC) + PCL-modified tumor cells (R+TM), B (cpm): PCL-modified tumor cells alone (TM), C (cpm): responders (PBMC) + unmodified tumor cells (R+T), D (cpm): unmodified tumor cells alone (T).

So, I_{prolif} = [(R+TM) – TM]/[(R+T) – T].

Table 1 Summary of patients and in vitro sensitization (IVS) data of autologous peripheral blood mononuclear cells (PBMC) stimulated by pressure/crosslinking (PCL)-modified or unmodified tumor cells. *R* responders, e. g. PBMC; *S* stimulators, e. g., tumor cells

Patient	Age (sex)	Cell viability (%)	Tumor cells (%)	R:S ratio	I_{prolif}	Response ($I_{prolif} \geq 2$ = +) ($I_{prolif} \leq 2$ = –)
Colon						
CC1	68 (M)	50	20	1:2	9.0	+
CC2	67 (M)	30	40	1:1	2.4	+
CC3	60 (F)	20	40	1:0.5	6.2	+
CC4	53 (M)	50	37	1:0.5	1.2	–
CC5	68 (F)	40	40	1:0.5	2.3	+
CC6	59 (F)	33	27	1:1	8.3	+
CC7	49 (F)	78	39	1:1	0.5	–
CC8	80 (M)	70	42	1:1	1.0	–
Mean ± SEM	63 ± 3.5	46.4 ± 7	35.6 ± 2.8		3.9 ± 1.2	5/8 (63%)
Lung						
LC1	63 (M)	50	50	1:2	3.1	+
LC2	76 (M)	86	20	1:0.25	1.0	–
LC3	66 (M)	90	25	1:0.25	1.6	–
LC4	64 (M)	90	30	1:0.5	<0[a]	–
Mean ± SEM	67 ± 3	79 ± 10	31 ± 6.6		1.9 ± 0.6	1/4 (25%)
Renal						
RCC1	73 (F)	82	34	1:0.5	16.0	+
RCC2	69 (M)	65	32	1:0.5	3.6	+
RCC3	72 (F)	70	46	1:0.5	2.5	+
RCC4	57 (M)	90	38	1:0.5	2.9	+
Mean ± SEM	68 ± 3.7	77 ± 5.7	38 ± 3		6.25 ± 3.3	4/4 (100%)

[a] Excessive thymidine uptake by tumor cells alone

Cytokine secretion in IVS

The change in cytokine secretion, Δ, was defined as $\Delta=[A-B]-[C-D]$ where *A*=concentration of IL-10 or IFNγ (pg/ml) secreted during 5-day IVS culture stimulated with PCL-modified tumor cells (R+TM), *B* = concentration of IL-10 or IFNγ (pg/ml) secreted during 5-day cultures of TM alone, *C* = concentration of IL-10 or IFNγ (pg/ml) secreted during 5-day IVS cultures stimulated with unmodified tumor cells (R+T), *D* = concentration of IL-10 or IFNγ (pg/ml) secreted during 5-day cultures of T alone.

So, $\Delta=[(R+TM)-TM]-[(R+T)-T]$

Cytopathology examination

A sample of $(0.2–1)\times10^6$ tumor cells in suspension was analyzed prior to PCL treatment for determining the percentage of tumor cells. Cells were smeared on a glass slide, stained and scored by a cytopathologist.

Statistical evaluation

Statistical analyses of population data were performed throughout using the Wilcoxon rank-sum test [10]. Significance was determined by two-tailed *P* values.

Results

Freshly resected colon, lung and renal tumors from 16 cancer patients were processed and modified by PCL as described in the Patients and methods. The patients' mean age distribution was similar in the three groups whereas the sex distribution was 4M/4F (colon), 4M/0F (lung) and 2M/2F (renal) respectively (Table 1). The histopathology was uniform within the colon and RCC groups, but more heterogeneous in the lung cancer group (2 adenocarcinomas, 1 small cell and 1 non-small-cell lung carcinomas). The actual amount of tumor cells present in the purified samples was typically in the 30%–40% range while cell viability was equally high for the lung and renal groups (79% and 77% respectively) but lower in the colon cancer group (46%).

The IVS assay was utilized to evaluate whether PCL treatment of tumor cells could stimulate the proliferation of autologous PBMC in vitro. The stimulation by PCL-modified tumor cells relative to unmodified ones was assessed by the proliferation index, I_{prolif} (see Patients and methods) where $I_{prolif} \geq 2$ was defined as significant on the basis of the historical experience with the mixed lymphocyte reaction assay to determine transplantation suitability [22]. The values for [(R+T) –T], [(R+TM) –TM] and R, are depicted in Fig. 1, and summarized in Table 1. In 63% (5/8) of colon and 100% (4/4) of renal carcinomas, PCL-modified tumor cells induced significant stimulation in autologous PBMC with a mean proliferation index of 3.9 and 6.25 respectively. Conversely in only 25% (1/4) of lung carcinomas did PCL treatment stimulate a significant response. It should be noted that in 2 cases of RCC, normal renal tissue was obtained from healthy portions of the resected kidneys and was used as unmodified/PCL-modified stimulators in the IVS assay. In both cases no lymphocyte response against

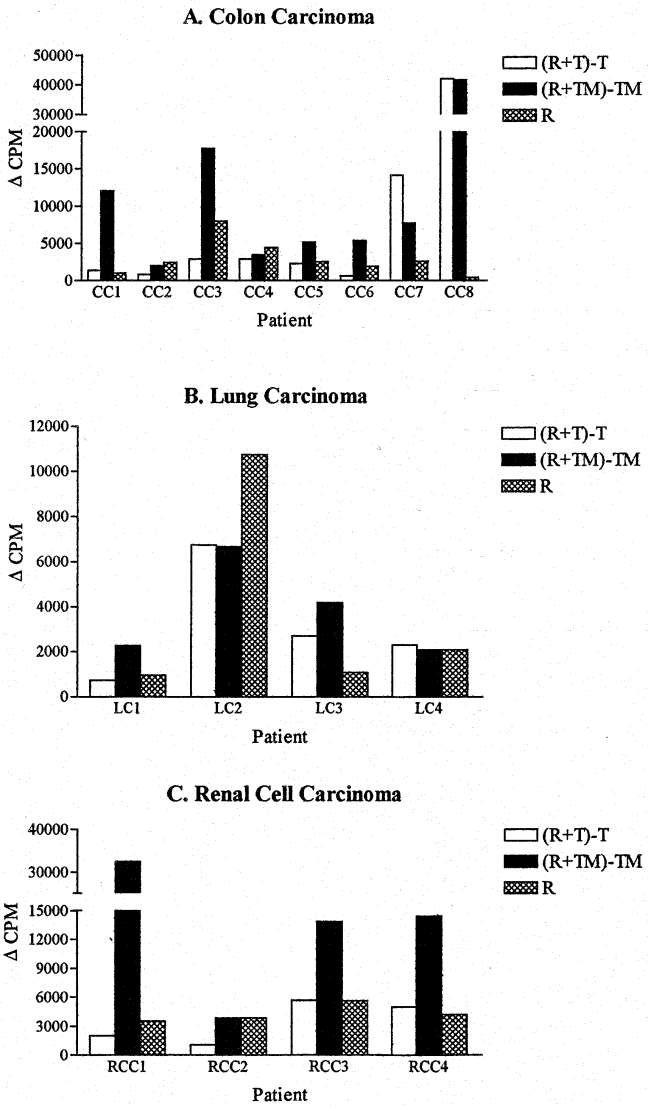

Fig. 1 Stimulation of PBMC by autologous tumor cells. Results are presented as net counts per minute (cpm) of [^{3}H]-Thymidine uptake in the IVS cultures (see Patients and methods)

unmodified or PCL-modified normal renal cells was observed (data not shown).

In order to evaluate the efficacy of the PCL-treated tumor cells on lymphocyte activation, we compared the raw triplicate radioactivity counts of all responder:stimulator ratios for both unmodified and modified tumor sets as [(R+T) $-T_{av}$] versus [(R+TM) $-TM_{av}$] for each disease type. T_{av} and TM_{av} refer to the average of the triplicate radioactivity counts of the unmodified and modified tumor cells alone for each ratio used. For this analysis, the Wilcoxon test was used to calculate the respective two-tailed *P* values, as summarized in Table 2. The confidence level of the results confirmed that the PCL modification of tumor cells increased their capacity to stimulate autologous lymphocytes.

The patterns of IL-10 and IFNγ secretion by autologous PBMC during the 5-day IVS cultures are shown in Table 3. The results indicate a clear trend of reduced IL-10 secretion in cultures stimulated by PCL-modified tumor cells in (4/7) colon, (3/4) lung and (2/3) renal cell carcinomas with an overall reduction observed in 9/14 patients (64%). Furthermore, an increase in IFNγ secretion was noted in (5/7) colon and (3/4) lung carcinomas; however, no such increase was observed in RCC patients (0/3). Overall, 8/14 patients (57%) demonstrated an increase in IFNγ secretion. There was no apparent correlation between changes in either IL-10 or IFNγ secretion during IVS and the proliferative index.

Discussion

In previous studies we have shown that PCL treatment of murine tumor cells increased their immunogenic potential both in vitro (IVS) as well as in vivo [20, 6, 21]. In the present study we further analyzed the immunostimulating effect of PCL on freshly prepared human tumor cells. Three different tumor types, colon, lung and renal carcinoma, were used as targets while proliferation and IL-10/IFNγ secretion of autologous lymphocytes served as output. Our results demonstrated that in 10 out of 16 patients (63%), exposure of tumor cells to PCL induced significantly higher autologous lymphocyte proliferation ($I_{prolif} \geq 2$) when compared to the proliferation against untreated tumor cells. In addition, population analysis (see Table 2) indicated that the increase in autologous lymphocyte activation in response to PCL treatment of the target tumor cells is statistically significant.

Various studies have shown that cytokines play a pivotal role in the regulation of immune response in a variety of pathological conditions. These regulating cytokines were shown to tip the balance towards either a Th1 or Th2-cytokine profile [5, 17]. For example, a number of infectious diseases appear to sustain their chronic status by stimulating an increase of systemic IL-10 and a decrease in IFNγ levels. Clonal expansion of CTL is thereby inhibited while B cells and antibody synthesis are stimulated. In the case of leishmaniasis, mice that display a high antibody titer coupled with expansion of Th2 cells develop chronic disease while animals that display an augmented Th1-mediated immune response against the pathogen recover spontaneously [13]. An analogous situation is seen in leprosy, where patients with persistent infection exhibit a predominantly humoral response with elevated Th2-type cytokines, while recovering patients display a strong cellular response associated with increased amounts of Th1 cytokines [24]. In another system, a high anti-HIV antibody titer in early infection coupled to elevated Th2 cytokines is a poor prognostic indicator for progression to AIDS [15]. Conversely, a minority of HIV-infected patients who mount a considerable Th1-mediated immune response appear to be less likely to progress to AIDS [5]. Recent evidence suggests that tumors may have adopted similar survival strategies by creating a predominant Th2 environment in situ to blunt cell-mediated immunity. A growing number of human tumors have been shown to secrete IL-10 constitu-

Table 2 Wilcoxon analysis of the raw radioactivity (cpm) data sets of [(R+T)–T_{av}] versus [(R+TM)–TM_{av}] by disease type. T_{av} and TM_{av} refer to the average of the triplicate radioactivity (cpm) counts of the unmodified and modified tumor cells alone for each ratio used. Values shown are the means (*n*) and the SEM

Disease	(R+T)–T_{av}	(R+TM)–TM_{av}	*P*
Colon carcinoma	8010 (33) 1952	10046 (33) 1954	0.029 (significant)
Lung carcinoma	2346 (15) 666	3496 (15) 565	0.042 (significant)
Renal cell carcinoma	3889 (24) 703	11840 (24) 2235	0.0024 (highly significant)

Table 3 Cytokine secretion in autologous PBMC cultures stimulated by unmodified (*T*) or PCL-modified (*TM*) tumor cells. *ND* not determined, *IL-10*, *IFN*γ

Patient	IL-10 (pg/ml)			IFNγ (pg/ml)			Notes
	(R+T)–T	(R+TM)–TM	Δ	(R+T)–T	(R+TM)–TM	Δ	
Colon							
CC1	190	0	–190	0	0	0	
CC2	1109	–557	–1666	0	2174	2174	The TM control secreted IL-10
CC3	720	182	–538	0	540	540	
CC4	20	74	54	0	370	370	
CC5							ND
CC6	0	0	0	–3813	–801	3012	The T and TM controls secreted IFNγ
CC7	–67	83	150	700	2140	1440	The T and TM controls secreted IL-10
CC8	40	0	–40	0	0	0	
							57% (4/7) IL-10 reduction and 71% (5/7) IFNγ increase
Lung							
LC1	868	484	384	–1226	–754	472	The T and TM controls secreted both IL-10 and IFNγ
LC2	635	307	–328	1300	2000	700	
LC3	274	160	–114	–982	–924	58	The T and TM controls secreted both IL-10 and IFNγ
LC4	–42	242	284	1594	–245	–1839	The T control secreted both IL-10 and IFNγ while the TM control secreted only IFNγ
							75% (3/4) IL-10 reduction and IFNγ increase
Renal							
RCC1	0	20	20	0	0	0	
RCC2							ND
RCC3	81	0	–81	0	0	0	The T control secreted IL-10
RCC4	640	198	–442	360	–280	–640	The T and TM controls secreted both IL-10 and IFNγ
							66% (2/3) IL-10 reduction and 0% (0/3) IFNγ increase

tively with only trace amounts of Th1 cytokines. These include melanoma [3], glioblastoma [14], bronchogenic carcinoma [26], malignant pleural effusion [4] and renal cell carcinoma [34]. It has been demonstrated that IL-10 can protect melanoma cells from tumor- and allo-specific cytotoxic T cells, as well as down-regulate HLA class I expression [16]. In addition, tumors can alter the Th state of their milieu by down-regulating the tumor surface expression of the costimulating B7-1 molecules, thereby causing suppression of the Th1 state [18, 32, 1].

On the basis of the above findings, levels of IL-10 and IFNγ secretions in the IVS assays were analyzed in order to determine the nature of the immunoregulatory response to unmodified and PCL-modified target tumor cells. Our results indicated that, in the majority of the patients tested, stimulation of autologous PBMC in culture by PCL-modified human tumor cells caused a significant reduction in IL-10 secretion (9 out of 14 patients, 64%) and increase in IFNγ secretion (8 out of 14 patients, 57%) relative to unmodified cells (Table 3). A notable exception was renal cell carcinoma, in which a decrease in IL-10 was observed but without increase in IFNγ. These results confirmed the efficacy of the PCL method in affecting the secretion of regulatory cytokines and raised the possibility that this procedure can selectively tip the cytokine balance in favor of a Th1-like response. Interestingly, as shown in Table 3, several of the untreated and treated tumor cells themselves secreted either IL-10, IFNγ, or both. In these cases there could be a bias in the cytokine determination caused by these tumor-produced secretions. Nonetheless, the trend even amongst those patients was in the direction of a Th2 to Th1 shift.

The molecular mechanism by which pressure-modified tumor cells become more immunogenic is not yet fully

delineated. Under high pressure (i.e. 122 MPa), the membrane lipid domains become considerably more rigid while the cytoskeleton polymers reversibly dissociate [20]. The ensuing changes in the topology of the cell surface are fixed by crosslinking with adenosine dialdehyde [20, 21]. The PCL-treated cells thus presumably present a higher level of MHC molecules, which is believed to be a key element in the observed increase in their immunogenicity. However, in addition to these passive processes, pressure can have a profound effect on protein synthesis, similar to other stress inducers, causing the transient induction of ()pressure-induced proteins“ [25]. In two separate cellular systems (*Escherichia coli* and osteosarcoma) the application of brief intense hydrostatic pressure resulted in the specific synthesis of heat-shock proteins, which could act as molecular chaperones [35, 12]. The latter were found to be essential for the transport of MHC/antigen complexes from the intracellular compartments to the cell surface [27] and have been shown to be down-regulated in several tumors [28]. Suto and Srivastava [30] have previously shown that a number of heat-shock proteins derived from tumor cells are potent immunogens by virtue of the tumor-associated antigens that are adsorbed onto them. As anticipated, several of these heat-shock proteins were found to be induced during the PCL of B16-BL6 tumor cells (A. Matsaev et al. in preparation). The character and immunological relevance of the generation of MHC-restricted cytotoxic T lymphocytes by PCL-modulated human tumor cells are under current investigation.

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