Immunotherapy of Cancer with Pressure Modified Cells

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Immunotherapy of human cancer has proven to be of limited clinical value thus far, largely because of its failure to overcome the multiple escape strategies deployed by tumor cells [1]. Renewed interest in cancer immunotherapy was stimulated in recent years by the identification of distinct tumor antigens that are capable of eliciting specific anti-tumor cytotoxic immune responses *in vitro* as well as *in vivo*. However, the availability of synthetic or purified tumor antigens may not in itself be sufficient to offer an improved therapeutic modality, since tumor antigens are numerous, metastatic tumor cells mutate frequently thereby altering their antigenic nature, and the immunogenic hierarchy of various tumor antigens is largely unknown [2].

The basic principles underlying tumor immunology that have evolved considerably over the past 40 years are straightforward:

- The normal immune system is rarely a significant barrier to tumor growth, although lymphocytes play both positive and negative roles in tumor growth and development
- Tumors bear antigenicity (i.e., the ability to induce antibody production) which not necessarily expresses immunogenicity (the ability to induce an overall immune rejection response)
- Manipulation of the immune system can lead to complete tumor eradication [3].

These statements raise a number of questions that lie at the heart of contemporary immunotherapeutic approaches to cancer therapy.

In the framework of current immunotherapeutic methodologies, prevailing strategies to elicit specific anti-tumor T cell responses include gene transfer into autologous tumor cells [4], vaccination with dentritic cell preloaded with tumor antigens [5], systemic or paracrine cytokine therapy with T cell helper type 1 cytokines (namely, interleukin-2 and granulocyte macrophage colony-stimulating factor) [6], vaccination with T cell epitopes derived from tumor proteins [7], and vaccination with tumor-derived heat shock proteins [8]. However, tumor cells can evade immune killing either by down-regulation or total absence of immunogenic surface molecules, including major histocompatibility class I, or by synthesis and secretion of molecules capable of inactivating cytotoxic effectors, as well as by defective capillary neovasculature around the malignant tissue that slows down or even prevents cytotoxic cell egress into the tumor mass [9]. *Therefore, the strategy of an efficient immunotherapeutic attack on metastatic cancer must be based on combined local and systemic approaches capable of coping simultaneously with such varied escape mechanisms.* The molecular and cellular approaches outlined above could theoretically increase the arsenal of immunotherapeutic techniques to direct against a neoplasm; but at least for now, most of these procedures are time consuming, costly and difficult to establish in a standard hospital setting. In contrast, straightforward physico-chemical modification of autologous tumor cells, aimed at transforming them into potent stimulators of an anti-tumor response, is a simple, efficacious and cost-effective method to prepare a whole-cell autologous – and perhaps in the future allogeneic – tumor vaccine.

In this vein, over the past decade, our group has brought hydrostatic pressure to bear on the augmentation of the immunogenicity of tumor cells. We have developed a method in which cells are exposed to high hydrostatic pressure in the presence of a slow-reacting biologically compatible impermeable cross-linker, adenosine diAldehyde. Hydrostatic pressure engendered rearrangement of proteins on the cell surface into heterologous clusters. This newly acquired immunogenic topology was then 'decorated' with free thiol groups by reducing the cell surface disulfide bonds with the biologically compatible reducing agent, *N*-acetyl-L-cysteine. Each of these methods by themselves, and especially their combination, induced a potent anti-tumor response against the native tumor. The pressure cross-linking and *N*-acetyl-L-cysteine methodologies are outlined in the following.

PCL can upgrade the immunogenicity of tumor cells

In the PCL protocol, tumor cells are exposed to high hydrostatic pressure in the presence of the cross-linker AdA to form a viable whole-cell vaccine capable of eliciting a strong anti-tumor immune response. This procedure was shown to be effective and practical for increasing the immunogenicity of both murine [10–13] and human tumor cells [14]. Specifically, cells are suspended in a physiologi-

PCL = pressure cross-linking
AdA = 2′-3′-Adenosine diAldehyde

cal buffer containing 10 mM AdA and are exposed to high hydrostatic pressure of up to 1,200 atmosphere. Under such pressure the cytoskeletal network, composed primarily of actin, microtubules and microfilaments, is almost completely dissociated into monomers [15]. Similarly, because phospholipid bilayers have appreciable compressibilities, pressure is able to order and condense the lipid bilayer. As a result, the application of high hydrostatic pressure causes many membrane proteins in the outer leaflet of the tumor cell surface to undergo both lateral and vertical displacement, and some of these proteins are probably shed into the surrounding medium due to excessive dissociation from the cytoskeletal anchorage [16]. Most of the remaining membrane proteins aggregate non-specifically to form clusters or 'patches' [17].

In addition to these physical phenomena, application of pressure stimulates the induction of biosynthesis of putative immunogenic heat shock-like proteins [18]. We recently detected an appreciable increase in the protein level of HSP90 and HSP gp96, as measured in Western blot analysis, in addition to an increase in the surface expression of HSP gp96 upon PCL treatment (Goldman et al, in preparation). Moreover, strong evidence is presented in the literature that it is precisely these HSPs that can serve as collectors and transporters of immunological peptides from the cytoplasm to the exterior plasma membrane [19]. Indeed, vaccination with HSP70, HSP90 and HSP gp96 were shown to elicit specific immunity against the tumor from which they were isolated [8]. Upon decompression, the proteins in these surface heterologous clusters, which are covalently cross-linked or interact non-covalently with one another, will remain associated as the cytoskeletal anchors reform. Taken together, this series of "active" and "passive" events results in an extensive projection of dormant and intracellular immunogenic proteins on the surface of the modified cells, thereby transforming them into putative targets for phagocytic antigen-presenting cells.

PCL modification of human tumor cells

After testing the method in murine tumor models, we employed the mixed lymphocyte tumor reaction, also known as *in vitro* sensitization, to determine whether PCL-modified cancer cells could stimulate proliferation of autologous peripheral blood mononuclear cells. We examined [14] the proliferative stimulation and cytokine secretion pattern in such autologous MLTR cultures using parental and PCL-modified tumor cells. These tumor cells were obtained from freshly resected tumor tissue of 16 patients with colon (n=8), lung (n=4) and renal (n=4) carcinomas. (All participants provided informed consent before entering the trial, which was approved by the internal review boards of the participating institutions). The results demonstrated that PCL-modified tumor cells promoted an increase in PBMC proliferation in 5 of 8 (63%), 1 of 4 (25%) and 4 of 4 (100%) colon, lung and renal cell carcinomas respectively. Fourteen of the above cultures were also analyzed for the secretion of IL-10 and interferon-gamma. Overall, a substantial decrease in IL-10 secretion was detected in 9 of 14 cultures (64%), while a reciprocal increase in IFN-γ secretion was noted in 8 of 14 cultures (57%). These results confirmed that PCL-modified human tumor cells of different etiologies, *in vitro,* can modulate the pattern of cytokines released from stimulated autologous lymphocytes.

Intercellular disulfide chemical bridging

The notion that reversible intercellular bridging between target and effector cells can strengthen specific immunological signals led us to investigate the effect of reversible intercellular disulfide bonds in provoking an anti-tumor immune response. Building on the platform of the PCL modification, we hypothesized that transient *intercellular chemical bridges* could be formed between immune effector and target cells, and could participate in stimulating an effective cellular immune response. An example of an intercellular connection is the amine-aldehyde, or Schiff base, bridge investigated by Rhodes et al. [20]. They showed that small aldehyde-containing molecules on the surface of target cells can conjugate continuously exposed protein amine groups on the surface of effector cells to deliver or amplify a stimulating signal. Encouraged by this finding, we searched for a natural analog to the Schiff base bridge and decided upon an intercellular disulfide bridge, based on several recent findings in the literature.

The importance of thiols (e.g., β-mercaptoethanol, cysteine) in enhancing the growth of cells in tissue culture has been known for over 20 years but the exact mechanism has been a matter of long-standing debate. Dröge et al. [21] demonstrated that the extracellular concentrations of thiols have a profound effect on T cell responses *in vitro,* and there are indications that cysteine may play a role in activating an immune response *in vivo*. A number of studies have further shown that the presence of free thiols in the tissue culture medium is essential for lymphocyte proliferation and activation. One interpretation of these data holds that extracellular thiols generate free surface sulfhydryl groups which could form intercellular disulfide bridges between target and effector in an analogous fashion to the Schiff base bridges mentioned above.

We selected the biologically compatible reducing agent, NAC, a mucolytic agent used in the treatment of respiratory disorders [22] for imposing reactive sulfhydryl groups

HSP = heat shock proteins
MLTR = mixed lymphocyte tumor reaction
PBMC = peripheral blood mononuclear cells
IL = interleukin
IFN = interferon
NAC = *N*-acetyl-L-cysteine

on the surface of tumor cells. Using NAC, we developed a novel combined cellular/systemic procedure for induction of an anti-tumor immune response against both unpressurized and PCL-treated cells. An example of the central role played by exposed surface thiols in augmenting a potent systemic immune response against experimental metastasis is illustrated in Figure 1. In mice immunized with unmodified 3LL-D122 murine Lewis lung carcinoma cells, the lung weight of 75% of them was above the normal 200 mg cutoff in contrast to only 25% for the NAC-modified group (modification including both tumor cell reduction and NAC i.v.). This experiment indicated that the combination of local immunization with NAC-treated tumor cells in addition to systemic NAC administration improved the systemic anti-metastatic activity considerably.

From a mechanistic point of view, the origin of this effect may lie in an increase of the half-life of the target cell-APC 'immunological synapse' [23], which could increase the rate of transfer of an immunological signal. Lanzavecchia and colleagues [24], who recently reviewed the kinetics of T cell receptor activation, point out that MHC/peptide complexes do not need to bind with high affinity in order to trigger the TCR. Strong TCR agonists are typically characterized by a Kd of 1–90 μM and half-lives of ~10 sec [25]. In line with these values, it is suggested that the reactive thiols on the surface of target cells form transient intercellular bridges, which prolong the time of interaction between the effector and the stimulator or APC and thereby enhance the rate of transfer of the specific immunological signal. This proposed mechanism could add another dimension to the kinetic proofreading model [26] by furnishing additional activation signals to T lymphocytes, which could aid in breaking the non-responsiveness of the immune system to disseminated metastatic foci.

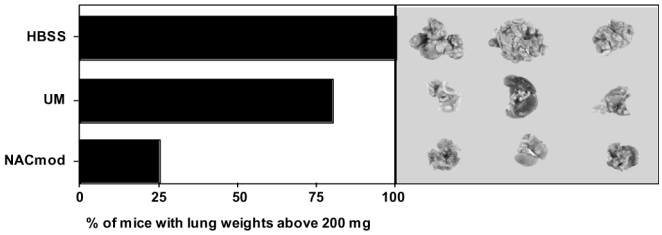

Figure 1. Assessment of lung metastasis in mice bearing lung tumors in an experimental metastasis assay. Groups of 9 to 10 C57BL6 mice were immunized i.p. 3 times, 1 week apart, with $2x10^6$ irradiated unmodified (UM) or NAC-modified D122 cells + NAC i.v. Seven days after the last immunization, mice were challenged with $2x10^5$ viable D122 cells i.v. After 3 weeks the mice were sacrificed and the lung weights were determined. The percentage of lungs above the upper normal lung weight (200 mg) is depicted. In comparing the NAC-modified (NACmod) D122 to Hank's buffered salt solution (HBSS), P=0.0007, and to unmodified D122 cells (UM), P=0.0085. Photographs of three representative lungs for each group are also presented.

APC = antigen-presenting cell
MHC = major histocompatibility complex
TCR = T cell receptor

The current state of the art: PCL+NAC modifications synergize to stimulate a potent systemic anti-tumor response

Combining the PCL and NAC methods, we assayed the putative synergism of the two-stage protocol both *in vitro* and *in vivo*. The most effective anti-tumor immune response was obtained with the two-pronged strategy composed of immunization with PCL+NAC modified cells and i.v. administration of NAC as a general T cell immunostimulant [21]. We then performed the experiment most closely resembling a clinical situation, namely the metastasis regression assay. In this model, mice were injected i.v. with 3LL-D122 cells and lung micrometastases were allowed to develop over 8 days. This waiting period was designed to simulate the tumor-bearing patient with disseminated disease who commences therapy. Subsequently, a series of immunotherapeutic regimens were administered, commencing on day 9. Figure 2 depicts the histological appearance of the lungs from mice immunized with unmodified cells as compared to mice immunized with PCL+NAC treated cells/NAC i.v. In the former, the tumor cells completely filled the alveolar spaces, whereas in the latter, the immune response induced by inoculation with PCL+NAC treated cells completely cleared the lung of metastases [27]. This assay presented strong evidence that the surface modification of the tumor cells by PCL+NAC, in the presence of systemic immunostimulation with NAC, induced the most potent anti-tumor immune response *capable of eradicating established metastases*.

The anti-tumor potency of the combined PCL+NAC protocol stands out in its efficacy and its applicability to the clinical setting. The advantage of this protocol was manifested in the quantitative measurement of delayed-type hypersensitivity to unmodified D122 cells after

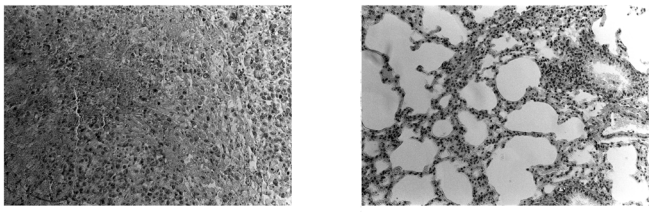

Figure 2. Photomicrographs of histological sections from lungs of the mice immunized with UM and PCL+NAC modified D122 cells. PCL synergizes with NAC to eradicate established lung metastases in a metastasis regression model. Groups of 10 C57BL6 mice were injected intravenously with $5x10^5$ viable D122 cells. Nine days later, when lung metastases were well established, various immunotherapeutic regimens were started. Twenty-five days after the third immunization the mice were sacrificed and the lung weights determined.

immunization with PCL+NAC modified cells, as shown in Figure 3. This immunization was sufficient to produce a significant DTH recall response to parental D122 cells *in vivo*. It is thus apparent that the PCL+NAC modification, combined with the NAC i.v administration, is capable of breaking the barrier of non-responsiveness of the immune system to the non-immunogenic D122 tumor cells by making the modified cells look "dangerous" to the immune system.

Why is it important for a tumor cell to look "dangerous"? In the past few years, Matzinger and colleagues [28] have developed the 'danger hypothesis,' which states that the immune system actually does not distinguish between self and non-self, but in fact protects against "danger" signals that are transmitted through positive and negative communications from an extended network of other bodily tissues, especially APCs. Furthermore, this hypothesis contends that if APCs cannot constitutively deliver co-stimulatory signals but are activated only when needed, they can serve as tissue-specific tolerizers and waste no energy on useless responses. In this scheme, *danger equals tissue destruction*. Since unprogrammed cell death does not usually occur by apoptosis, it could serve as a clue to local APCs that unplanned destruction (necrosis) is occurring. In contrast, other groups have recently shown

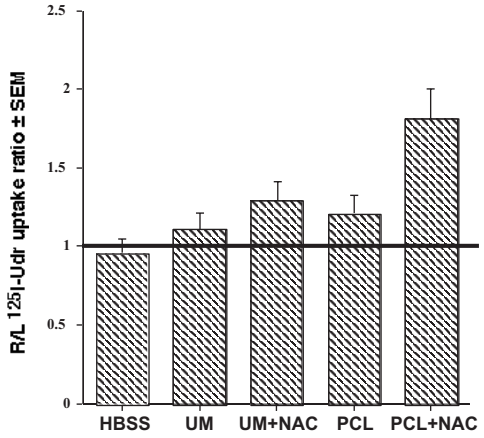

Figure 3. Immunization with PCL+NAC modified D122 cells stimulated a DTH reaction against UM D122 targets. On week 1, groups of 5 to 6 mice were injected i.p. with $2x10^6$ UM, UM+NAC, PCL or PCL+NAC irradiated D122 cells; those mice receiving the UM+NAC and PCL+NAC treatment were then boosted 3–4 days later with a 200 µl injection of 15 mM NAC i.p. This immunization protocol with NAC boosting in the UM+NAC and PCL+NAC groups was repeated twice more, on weeks 2 and 3. Mice immunized with UM or PCL cells alone were administered phosphate-buffered saline at the time of NAC boosting. A quantitative DTH test was performed [40] where UM D122 cells were injected into the right ear (R) and buffer into the left ear (L). The right/left ear (R/L) uptake (mean ± SEM) of 5-[^{125}I]-Iodo-2'-deoxyuridine, a quantitative measure of the anti-tumor T cell response, is presented. In comparing the PCL+NAC immunization to HBSS, *P*=0.0081, to UM, *P*=0.0255, to UM+NAC, *P*=0.0866 and to PCL, *P*=0.0475. (Adapted from ref. 27 with permission from *Cancer Research*)

DTH = delayed-type hypersensitivity

that dentritic cells can acquire antigens from apoptotic cells and induce class I-restricted cytotoxic T lymphocyte responses [29].

Based on what we mentioned above regarding the putative involvement of HSP in the PCL mechanism, we hypothesize that PCL+NAC cells in the inoculum present immunogenic HSPs in addition to other immunogenic components on their surfaces, and possibly leak such HSP-peptide complexes into their immediate vicinity (Goldman et al., in preparation). Given this supposition, the immunogenic HSP products, presented and possibly shedded from PCL-modified cells, could serve as activation signals when taken up by APCs such as dentritic cells either from the interstitial fluid space (micropinocytosis) or from phagocysed modified cells [30]. These activated APCs responding to these "danger signals" could then present these processed antigens to both $CD8^+$ and $CD4^+$ T cells in the draining lymph node, perhaps by means of sequential 'licensing' of dentritic cells [31]. Circulating NAC could be simultaneously stimulating these T cells in a positive feedback loop, thereby augmenting their capacity to respond to the remaining metastatic cells. A schematic diagram of the proposed mechanism of the PCL+NAC modification is presented in Figure 4. Further clarification of the role of HSP and dentritic cells in the PCL immunostimulating mechanism is currently being pursued in our laboratory. In summary, we therefore propose that, the regimen of *local* immunization with PCL+NAC modified tumor cells and *systemic* NAC administration together meets the stringent criteria of a "multivalent" tumor vaccine that may eventually be

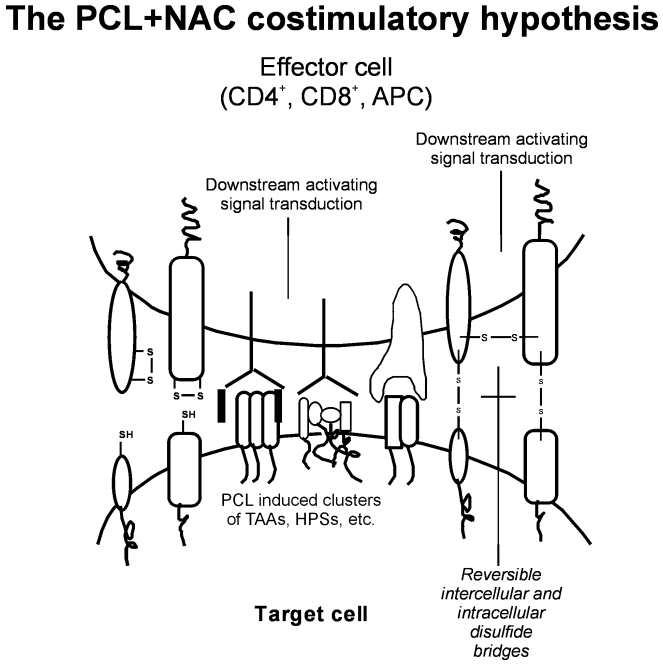

Figure 4. Schematic diagram of the PCL+NAC mechanism

applied as an immunotherapeutic treatment regimen in selected human cancers.

Future directions

The PCL+NAC method is an effective, straightforward and economically feasible procedure that can generate an autologous multivalent whole-cell tumor vaccine capable of propagating a potent anti-tumor immune response. The equipment needed to carry out this procedure is not expensive and is simple to operate in a standard hospital research facility equipped for the manipulation of human tissue under sterile conditions. Moreover, only a short training is necessary to gain proficiency in the procedure. The pre-clinical experiments carried out with human cancer cells have demonstrated the potential of applying the technique as an immunotherapeutic regimen in human cancer [14].

The anti-tumor potency of the combined PCL+NAC protocol is striking in its efficacy and its applicability to the clinical setting. The anti-tumor activity of this novel innocuous methodology compares well with other experimental immunotherapeutic approaches [32,33]. However, one of the inherent weaknesses of the PCL+NAC, like any other autologous tumor cell-based vaccine, is the lack of sufficient tissue for operation. Were it possible to employ totally characterized, standardized human tumor cell lines as a source for first-line or booster tumor cell-based vaccines, the shortage of material would not present a problem. In our opinion, the future direction of modified tumor cell-based vaccines will tend toward the development of well-characterized, standardized human tumor cell lines that could form the basis of allogeneic "poly-vaccines" [34]. In such a vaccine, PCL+NAC treatments are expected to promote the inherent immunogenicity to an extent similar to that observed with autologous cells. Preliminary human studies with allogeneic vaccines are currently being conducted by other groups for melanoma [35], as well as in cases of colorectal carcinoma [36], pancreatic adenocarcinoma [37], relapsed neuroblastoma [38] and acute lymphoblastic leukemia [39]. In the long term, if certain types of human tumors display a sufficient number of common tumor antigens such that immunization with-defined allogeneic cell lines can induce a potent anti-tumor response, we may envisage prophylactic immunization of high risk populations with PCL+NAC based allogeneic poly-vaccines, most notably in melanoma and breast cancer.

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