Tumor Cell Lysate-pulsed Human Dendritic Cells Induce a T-Cell Response against Pancreatic Carcinoma Cells: an in Vitro Model for the Assessment of Tumor Vaccines

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells and play an important role in T cell-mediated immunity. DCs have been shown to induce strong antitumor immune responses in vitro and in vivo, and their efficacy is being investigated in clinical trials. Compared with vaccination strategies directed against a single tumor antigen, tumor-cell lysate as the source of antigen offers the potential advantage of inducing a broad T-cell response against multiple known, as well as unknown, tumor-associated antigens expressed by the individual tumor. We used pancreatic carcinoma cell lines to develop an in vitro model for monitoring T-cell responses induced by lysate-pulsed DCs. Monocyte-derived DCs of HLA-A2+ donors were pulsed with lysate generated from the HLA-A2+ pancreatic carcinoma cell line Panc-1. In some experiments, the immunogenic protein keyhole limpet hemocyanin (KLH) was added to the lysate. Subsequently, the antigen-loaded DCs were activated with tumor necrosis factor-α and prostaglandin E2. Autologous mononuclear cells were cocultured with DCs in the presence of low-dose interleukin (IL-2) and IL-7 and were restimulated weekly with new DCs. High levels of IL-12 and IFN-γ could be detected in the supernatants, indicating a T-helper type 1-type immune response. This cytokine profile was associated with the expression of the activation marker CD69 on both T helper and CTLs and with an antigen-induced proliferative T-cell response. After 4 weeks, CTL-mediated cytotoxicity was assessed. Tumor cell lysis was specific for Panc-1 tumor cells and was MHC class I-restricted. Cytokine secretion, CD69 expression of T cells, and antigen-induced T-cell proliferation correlated with the cytotoxic activity and were more pronounced when KLH was added to the lysate. This is the first study to show that T cells specific for pancreatic carcinoma cells can be generated in vitro by lysate-pulsed DCs and that the T-cell response can be enhanced by KLH. This in vitro model can be applied to compare different strategies in the development of DC-based tumor vaccines.

INTRODUCTION

Current strategies in the treatment of metastasized pancreatic carcinoma offer little hope for a cure and have only slight impact on survival (1). Therefore, new treatment modalities are warranted for this type of cancer. Efforts in the immunotherapy of malignant disease concentrate on the induction and enhancement of immune responses against tumors. A promising approach is the use of DCs,4 which are highly effective antigen-presenting cells with the unique capability of inducing primary immune responses against tumor-associated antigens (2). Animal studies (3, 4) and human cancer trials (5–9) have shown that specific T-cell responses against tumors as well as tumor regression can be achieved with vaccines based on DCs. Potential targets for the immunotherapy of pancreatic carcinoma are antigens such as carcinoembryonic antigen (10, 11), HER-2/neu (12, 13), MUC-1 (14–17), mutant ras (18, 19), p53 (20, 21), and gangliosides (22). However, vaccinating against a single antigen has disadvantages, because it is unknown which of the identified antigens have the potential to induce an effective antitumor immune response. Furthermore, immunity against a single antigen may be ineffective in tumors with heterogeneous cell populations and carries the risk of inducing tumor antigen escape variants (7). In addition, this strategy is restricted to those patients with a specific HLA type.

The use of unfractionated tumor-derived antigens in the form of tumor cell lysates or whole tumor cells circumvents these disadvantages. Tumor lysates contain multiple known as well as unknown antigens that can be presented to T cells by both MHC class I- and class II-pathways (4, 23, 24). Therefore, lysate-loaded DCs are more likely to induce a polyclonal expansion of T cells, including MHC class II-restricted T-helper cells. These have been recognized to play an important role in the activation of CTLs, probably the most important cells in effecting an antitumor immune response (25). The generation of CTL clones with multiple specificities may be an advantage in heterogeneous tumors and could also reduce the risk of tumor escape variants. Furthermore, lysate from the autologous tumor can be used independently of the HLA type of the patient. A major drawback of unfractionated tumor antigens is the possibility of inducing an autoimmune reactivity to epitopes that are shared by normal tissues (26). However, in clinical trials using lysate or whole tumor cells as the source of antigen, no clinically relevant autoimmune responses were detected (6, 8, 27).

For the development of an effective tumor vaccine based on DCs, the influence of experimental parameters on the immune response remain to be determined, such as the optimal source and subtype of DCs, the choice of the tumor antigen preparation, methods for effectively introducing antigens into MHC class I- and II-processing pathways, and the use of activators and adjuvants. Because not all of these parameters can be optimized in controlled clinical trials, there is a need for in vitro models to address these questions.

In this study, we present an in vitro model designed in our laboratory to test different strategies for the development of a therapeutic vaccine against pancreatic carcinoma based on tumor lysate-pulsed DCs. Several parameters such as the cytokine profile, tumor antigen-induced T-cell proliferation, expression of T-cell activation markers, and cytolytic T-cell activity were assessed to characterize the immune response.

MATERIALS AND METHODS

Reagents. Recombinant human cytokine GM-CSF was purchased from Novartis (Basel, Switzerland), IL-4 from Promega (Madison, WI), IL-2 and IL-7 from Strathman Biotech (Hannover, Germany), and TNF-α from R&D Systems (Wiesbaden, Germany). PGE2, KLH, and FITC-dextran were obtained from Sigma Chemical Co. (Munich, Germany). [3H]Thymidine was purchased from Amersham Buchler (Freiburg, Germany) and [35S]sodium chromate from NEN Life Sciences (Zavantem, Belgium).

Cell Culture. Cell cultures from human peripheral blood mononuclear cells were maintained in RPMI 1640 culture medium (Biochrom, Berlin,
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RESULTS

Testing of the Tumor Cell Lysates. Generation of monocyte-derived DCs was highly reproducible. After a single adhesion step and 5 days of culture in the presence of GM-CSF and IL-4, the yield of DCs was approximately 8–15% of the isolated peripheral blood mononuclear cells. DCs presented as clusters of large cells with stellate morphology. DCs strongly incorporated FITC-dextran, expressed CD54, MHC class II, and low levels of CD80 and CD86 (Fig. 1). Upon a 48-h activation period with TNF-α and PGE₂, DCs were characterized by a low endocytotic activity for dextran, high expression of costimulatory molecules, MHC class II and CD83 (Fig. 1), secretion of IL-12 (Fig. 3), and an enhanced T-cell stimulatory capacity (Fig. 2; unpulsed).

On day 5, DCs were pulsed with lysate from the three pancreatic carcinoma cell lines AsPaC-1, Panc-1, and Capan-1 at protein concentrations ranging from 30–480 µg/ml. Viability of lysate-pulsed DCs was assessed by propidium iodide-staining and the exclusion of trypan blue dye. Lysate concentrations of 240 µg/ml or more were found to be toxic, whereas 120 µg/ml (equivalent to 1.3 tumor cells/DC) or less did not influence viability (data not shown).

Next, we assessed the T-cell stimulatory capacity of lysate-pulsed DCs in an allogeneic mixed lymphocyte reaction. Interestingly, the pulsing of DCs with lysate of three pancreatic carcinoma cell lines had different effects on DC function (Fig. 2). Lysate of AsPaC-1 activated

Fig. 1. Decrease of endocytotic activity and changes in phenotype of DCs by stimulation with TNF-α and PGE₂. On day 5 of culture, DCs are highly endocytotic (A, uptake of FITC-labeled dextran) and express low levels of CD80 and CD86 (B) and intermediate levels of MHC class II (C) and CD54 (D). After a 48-h incubation in the presence of TNF-α and PGE₂, DCs lose their ability to incorporate dextran, up-regulate CD80, CD86, CD54, and MHC class II, and express the maturation marker CD83 (E).
DCs, which was reflected by an increased expression of costimulatory molecules and MHC-II as well as an enhanced T-cell proliferation rate. However, CD83 expression was not induced. In contrast, lystate of Capan-1 completely inhibited the T-cell stimulatory capacity of DCs, without altering the surface marker expression. Subsequent activation of DCs with TNF-α and PGE₂ induced maturation, but the DCs were still dysfunctional. Pulsing DCs with lystate of Panc-1 did not significantly influence surface marker expression or T-cell proliferation rates, and an activation with TNF-α and PGE₂ markedly improved their T-cell stimulatory capacity.

For the long-term cocultures of DCs with T cells, we used lystate of the HLA-A2⁺ cell line Panc-1 to load immature DCs of HLA-A2⁺ donors at a final concentration of 120 μg of protein/ml. DCs were subsequently activated with TNF-α and PGE₂ for 24 h.

Lysate-pulsed DCs Induce a Th1 Cytokine Profile in Cocultures with Autologous MNC. Nonadherent MNC cocultured with autologous DCs were weekly restimulated with DCs. DCs were either unpulsed or pulsed with lystate in the absence or presence of KLH. High levels of IL-12 were detected in the supernatants of MNC cocultured with mature DCs (Fig. 3). IL-12 secretion was enhanced in cultures in that antigens from tumor lysates were presented by DCs. A further increase was observed when KLH was added to the lystate. IFN-γ secretion was strongly dependent on the presentation of tumor antigens by DCs, again with higher levels detectable if KLH was added. In all of the cultures, IL-4 was near or below the detection limit.

T Cells Cultured with Lysate-pulsed DCs Up-Regulate CD69. CD69 expression is induced early in T-cell activation after stimulation of the T-cell receptor (29) and is expressed by antigen-specific T cells secreting IFN-γ and TNF-α (30, 31). From the same coculture as above, T cells were removed 48 h after the second stimulation with DCs, and the expression of CD69 was analyzed by flow cytometry. Whereas T cells cultured in the absence of DCs did not express this activation marker (data not shown), 2.9% of the CTLs (CD8⁺) and 3.9% of the T-helper cells (CD4⁺) expressed CD69 when cocultured with mature DCs (Fig. 4). Coculture with tumor lysate-pulsed DCs enhanced CD69 expression of both CTLs and T-helper cells to 8.3% and 9.4%, respectively. If KLH was added to the lystate, 17.1% of the CTLs and 17.8% of the T-helper cells expressed CD69.

T-Cell Proliferation Is Enhanced after Re-exposure to Tumor Antigens Presented by DCs. Assuming that lysate-pulsed DCs prime naïve T cells toward tumor antigen-specific T cells and that these cells predominantly proliferate when exposed to antigen, we assessed the proliferation of MNC from the cocultures after restimulation with unpulsed or lysate-pulsed DCs. Incorporation of [³H]thymidine into proliferating cells was assessed during the last 18 h of a 4-day coculture. MNC from the coculture with lysate-pulsed DCs showed an increase in proliferation rates after rechallenge with antigens presented by lysate-pulsed DCs compared with unpulsed DCs. If KLH was added to the lystate, a restimulation with lysate-pulsed DCs (no KLH) induced a further increase in proliferation (Fig. 5). Proliferation rates showed a good correlation with CD69 expression of T cells in all donors.

Lysate-pulsed DCs Induce a MHC Class I-restricted Tumor Cell Lysis. Next, we investigated whether MNC from the cocultures were able to specifically recognize and kill Panc-1 tumor cells. After four weekly stimulations with unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs, we removed the nonadherent cells from the cocultures and incubated them with ⁵¹Cr-labeled Panc-1 tumor cells (HLA-A2⁺). MNC from the coculture with lysate-pulsed DCs were able to specifically lyse Panc-1 tumor cells (Fig. 6). Lytic activity was enhanced in the cocultures with lysate- plus KLH-pulsed DCs. KATO-III was lysed to a minor extent only, and the lysis was comparable with that of Panc-1 by MNC that had been cocultured with unpulsed DCs. To test whether tumor cell lysis was MHC class I-restricted, we preincubated the tumor cells with a MHC class I-blocking antibody. This substantially reduced tumor cell lysis (Fig. 7). To exclude unspecific lysis mediated by NK cells, which contributed approximately 2–3% of the MNC at the end of the coculture, cytotoxicity directed against NK cell-sensitive K562 cells was shown.

Fig. 3. DCs pulsed with Panc-1 tumor cell lystate induce a Th1 type cytokine profile. Forty-eight h after the second stimulation of MNC with unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs, supernatants were collected and analyzed for secreted IL-12, IFN-γ, and IL-4 by ELISA. Data are shown as means ± SE of three experiments.
was assessed, but no significant lysis could be observed (<5%; data not shown).

**DISCUSSION**

This study demonstrates the generation of an effective CTL response against pancreatic carcinoma cells by repeated in vitro stimulation of T cells with tumor lysate-pulsed DCs. Immature monocyte-derived DCs were used for pulsing with lysate because of their high phagocytic capacity (32). Subsequently, DCs were matured because DC-mediated immune responses are more effective if DCs receive an activation signal. This can be microbial products, such as lipopolysaccharide or unmethylated CpG motifs mimicking bacterial DNA (33), inflammatory mediators, such as TNF-α, PGE2, IL-1β, IL-6, monocyte-conditioned media, and extracellular ATP (34, 35), or T cell-derived signals, such as CD40 ligand (36). Matured DCs up-regulate costimulatory molecules, secrete the T-cell differentiation factor IL-12, and present antigens more effectively because of increased phenotypic stability and extended half-life of MHC class I- and II-molecules (37). Furthermore, immature DCs bear the danger of inducing nonproliferating, IL-10-producing T cells, whereas mature DCs propagate the development of Th1 cells (38). In respect of clinical applicability, we activated DCs with a combination of TNF-α and PGE2, which has been shown previously (39) to induce a mature phenotype with high expression of MHC class II, adhesion and costimulatory molecules, the secretion of IL-12, and enhanced T cell-stimulatory capacity.

Recently (40, 41), it has been postulated that DCs exposed to stressed or necrotic tumor cells mature spontaneously without the need of other activators. However, it cannot be excluded that this effect was caused by infection of the cell lines with *Mycoplasma* (42). In our hands, pulsing DCs with lysate from pancreatic carcinoma cell lines did not induce maturation as assessed by the lack of CD83 expression, but affected phenotype and T-cell-stimulatory capacity of DCs depending on the carcinoma cell line that was used. Enhanced T-cell stimulation of AsPc-1-pulsed DCs correlated with an increased surface expression of MHC class II and costimulatory molecules. Pulsing DCs with lysate of Panc-1 had no influence on surface marker expression or T-cell-stimulatory capacity, whereas lysate of Capan-1 completely inhibited DC-mediated T-cell proliferation. This inhibition did not correlate with the phenotype of the DCs and could not be reversed by subsequent activation with TNF-α and PGE2. Because...
with lysate-pulsed DCs. Thus, IFN-γ production was lower in cultures with unpulsed DCs compared with high amounts in the cocultures. However, this in vitro system does not allow defining the precise antigen specificity of the CTLs, and it cannot be excluded that other than tumor-specific antigens as well as additional MHC molecules could be involved in the recognition of the tumor cells. Unspecific lysis mediated by NK cells, contributing less than 3% to the MNC in the cocultures, is unlikely because no significant lysis of NK-sensitive K562 cells was observed. In conclusion, CTLs generated by repeated in vitro stimulations with lysate-pulsed DCs were able to specifically recognize and kill pancreatic carcinoma cells.

Adjuvants may play a major role in the induction of immune responses mediated by DCs (46, 47). The adjuvant properties of the highly immunogenic protein KLH have been used in vaccination trials either by coadministration of KLH with the DC vaccine (6, 9, 27) or by linkage of KLH to the relevant antigens (48–50). We observed that loading DCs concomitantly with lystate and KLH enhanced the T-cell response against tumor antigens. Higher levels of IL-12 and IFN-γ were detected in the supernatants, and an increased fraction of both CD4+ and CD8+ lymphocytes expressed the activation marker CD69. Furthermore, T cells from the cocultures with lysate- plus KLH-pulsed DCs proliferated to a higher extent when rechallenged with tumor antigens presented by DCs, and they lysed tumor cells more efficiently. These observations favor the concept that KLH can serve as a helper antigen augmenting tumor-specific immune responses (51). The simultaneous presentation of helper antigens and tumor antigens by DCs may lead to side-by-side activation of T cells specific for the helper and the tumor antigen, thereby providing a feedback signal for the antigen-presenting cell via CD40 ligation (36). The up-regulation of costimulatory molecules as well as secretion of IL-12 by the DCs could enhance the immunogenicity of tumor antigens that are otherwise not recognized by the immune system. Another mechanism involved could be a direct activation of CTLs by CD40-activated DCs (52). From our data, we conclude that KLH cannot be used only as a tracer molecule in vaccination trials, but also as an adjuvant for vaccines based on tumor lysate-pulsed DCs.

In summary, we demonstrated that CTLs specific for pancreatic carcinoma cells can be generated in vitro with tumor lysate-pulsed DCs. Vaccination of pancreatic carcinoma patients with lysate-pulsed DCs might offer an additional therapeutic option for these patients. Using autologous tumor cells for the generation of lysates may enable the immune system to generate a monoclonal T-cell response against a broad spectrum of tumor antigens expressed by the individual tumor. Furthermore, assuming that pancreatic carcinoma cells share common rejection antigens allogeneous carcinoma cell lines could be used as a source of antigen for patients who are not eligible for surgery (53). However, the feasibility of this approach still needs to be addressed in additional experiments.

We present an in vitro model applicable to improve and to monitor tumor vaccines based on DCs pulsed with unfractionated tumor antigens. We are currently investigating the influence of several parameters on the antitumor T-cell response such as maturation status of DCs, tumor antigen preparation (e.g., unfractionated cell lysate, apoptotic tumor cells, apoptotic bodies, or tumor RNA), and the use of adjuvants to develop a DC-based tumor vaccine against pancreatic carcinoma.

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