Prednisolone Reduces TNF-α Release by PBMCs Activated with a Trifunctional Bispecific Antibody but not their Anti-tumor Activity

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Abstract. *Background: New adjuvant immunological therapies, that selectively redirect effector cells towards tumors, are currently under development. These strategies include trifunctional bispecific antibodies (trAb) as promising tools for the elimination of disseminated tumor cells and micrometastases. To date, these chimeric molecules have demonstrated their antitumor potential mainly in vitro. Here, trAb-activated peripheral blood mononuclear cells (PBMCs) displayed considerable antitumor activity, accompanied by the release of cytokines, which contributed to the antitumor activity but, on the other hand, may evoke serious limiting side-effects in vivo, demanding therapeutic interventions. Materials and Methods: The antitumor activity and cytokine release by trAb-activated PBMCs were studied in co-cultures with multicellular tumor spheroids (MTS), which represent a three-dimensional in vitro model for solid tumors, especially non-vascularized micrometastases. The glucocorticoid prednisolone was tested for its influence on the release of TNF-α and the activity of PBMCs. Results: It was shown that PBMCs, which were stimulated with a trifunctional bispecific antibody, BiUII, displayed an excellent antitumor activity, resulting in complete disintegration of the MTS. Also, it was demonstrated that prednisolone significantly reduced the release of TNF-α, without impairing the antitumor activity of BiUII-activated PBMCs. In contrast, unspecific killing was reduced, as* demonstrated with an identical trAb (Bi48), which recognizes an antigen absent from the target cells. Conclusion: The in vivo application of bispecific antibodies for adjuvant tumor therapies may be limited by the manifest activation of immune effectors, accompanied by overwhelming cytokine release. Glucocorticoids, like prednisolone, may effectively reduce cytokine release without impairing the antitumor activity of trAb-activated immune cells.

Occult tumor cells, or micrometastases of solid tumors, remaining after primary surgery, are the origin for local recurrence and metastases and are, thus, the most frequent cause of death in cancer patients. Immunological interventions, aimed at the recognition and subsequent destruction of disseminated tumor cells directly after surgery, offer the best therapeutic chance for a cure since only small numbers of malignant cells are present during the latent phase of minimal residual disease.

Antibodies, either monospecific or bispecific, are tools frequently used for adjuvant treatment in this situation. Bispecific antibodies are synthetic chimeric molecules that, by definition, recognize target cells *via* a specific antigen and simultaneously activate one or several classes of immune effector cells (1-4). Trifunctional antibodies (trAb) are chimeric intact antibodies, which specifically recognize target cells and simultaneously activate different classes of immune effectors, yielding optimal immune responses (5-9). This activation is accompanied by the release of perforin (10), granzymes A and B, and cytokines like TNF-α (11), resulting in the killing of the target cells (12).

Multicellular tumor spheroids (MTS) represent an attractive model of intermediate complexity between cancer cell lines growing as monolayers *in vitro* and solid tumors *in vivo*. Compared to conventional monolayers, MTS resemble

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the *in vivo* situation more closely with respect to cell shape, cell-cell interaction and environment. Consequently, MTS are regarded as a valid and reproducible surrogate system, especially for non-vascularized micrometastases (13).

The trAb used in this study is comprised of two potent Ig-subclasses, mouse IgG2a and rat IgG2b. The trAb recognizes human carcinoma cells *via* the tumor-associated antigen EpCAM (14) and activates T lymphocytes *via* CD3. In addition, the trAb also binds Fcγ-R+ accessory cells *via* its complete Fc part. We have recently demonstrated, *in vitro,* the excellent antitumor activity of this antibody and the concomitant abundant synthesis and release of cytokines (8, 9, 11, 12), which may not be acceptable in systemic applications *in vivo*. The *in vivo* application of trAb may result in the release of large amounts of the pro-inflammatory cytokine TNF-α, thus requiring treatment interventions.

Here, the MTS model was used to investigate the impact of a glucocorticoid, prednisolone, on the antitumor activity of peripheral blood mononuclear cells (PBMCs), activated by the trAb, as well as on the TNF-α levels, released by these PBMCs. Prednisolone significantly reduced TNF-α without having measurable effects on tumor cell killing, at least in this experimental setting.

Materials and Methods

Antibodies. BiUII consists of an arm, which specifically binds the human EpCAM molecule and an arm, which specifically binds to human CD3. The EpCAM arm is derived from a murine hybridoma (C215) and is of an IgG2a isotype. The CD3 arm is derived from a rat hybridoma (26II6) and is of an IgG2b isotype. The trAb Bi48 is identical to BiUII, but recognizes the antigen E48 (15) instead of EpCAM. The trAb BiLu is identical to BiUII, but recognizes the murine CD3 molecule instead of the human CD3. All trAb are derived from fusions of two hybridomas and were purified by different chromatographic steps, as described (6). All trAb were used at a final concentration of 10 ng/ml.

Preparation of human mononuclear cells from peripheral blood. Heparinized peripheral blood was drawn from healthy volunteers and PBMC were prepared by Ficoll density centrifugation, according to standard protocols. In brief, the peripheral blood was isolated by venipuncture and diluted with phosphate-buffered saline (PBS). This solution was layered over a Ficoll cushion and centrifuged at 800 xg for 20 min at room temperature; mononuclear cells were isolated and washed twice with PBS before they were resuspended in standard cell culture medium (DMEM).

Cell lines and multicellular tumor spheroids. FaDu and UM-SCC 22A are permanent human tumor cell lines derived from hypopharyngeal tumor biopsies. Both cell lines are well known in the literature dealing with head and neck cancer. Both lines display high levels of surface EpCAM, but are negative for E48, as revealed by FACS using specific antibodies (data not shown). MTS were generated by plating 3000 cells, resuspended in 100 μl

DMEM, onto agarose (1% in DMEM) in 96-well cluster plates. The plates were incubated at 37°C in 5% CO_2. 6×10^4 PBMCs (E:T ratio=20:1) were added 24 h later when the spheroids had formed spontaneously. To test for viable tumor cells, MTS were replated in flat-bottomed cluster plates, where they grew as a monolayer culture. The outgrowth of cells was followed by light microscopy for up to 7 days after replating. Prednisolone-21-hydrogensuccinate (Solu-Decortin H; SDH; Merck Pharmaceuticals) was added to a final concentration of 5 μg/ml.

FACS analysis. Single cell suspensions from MTS were generated by trypsin digestion. The cells were incubated for 30 min on ice with the primary monoclonal EpCAM-antibody, C215, in PBS/5% fetal calf serum (FCS), washed twice in PBS and then incubated with a FITC-labelled secondary antibody (anti-mouse IgG) for another 30 min. Following two final washing steps, flow cytometry was performed using a FACSCalibur cytometer and the CellQuest analysis software (Becton Dickinson, Heidelberg, Germany).

TNF-α bioassay. Biologically active TNF-α in the cell culture supernatants was measured using the TNF-α-sensitive indicator cell line L929, as described (16). In brief, L929 cells were seeded in triplicate (2.5×10^4/well) and grown to confluence. Supernatants were added in serial dilutions and the cells were incubated for 24 h. The results were measured using a conventional MTT assay, as described (17). Recombinant human TNF-α served as standard and actinomycin D (Sigma, 1 μg/ml) was added to enhance sensitivity.

Prednisolone (SDH). SDH was dissolved in PBS and used at a final concentration of 5 μg/ml.

Results

SDH reduces TNF-α release of trAb-activated PBMCs in vitro. Activation of PBMCs with trAb leads to the synthesis and release of cytokines, including TNF-α, *in vitro* (9, 11). TNF-α is released in many inflammatory conditions and excessive systemic amounts evoke serious adverse events. We, therefore, wanted to investigate whether a glucocorticoid, prednisolone (SDH), reduces TNF-α release by trAb-activated PBMCs. To this end, MTS were generated from the two cell lines FaDu and 22A and co-cultured with PBMCs. Finally, trAb (final concentration 10 ng/ml) and/or SDH (final concentration 5 μg/ml) was added and the system incubated for 24 h at 37°C. Then, the supernatants were removed and serial dilutions were added to the TNF-α-sensitive indicator cell line L929, essentially as described (16). It became clear that SDH significantly reduced the release of TNF-α, *i.e.* by 82% with FaDu-MTS and by 76% with 22A-MTS (Table I).

SDH does not inhibit killing of target cells by BiUII-activated PBMCs. In order to investigate whether the addition of SDH influences the antitumor activity of PBMCs in our experimental setting, FaDu- and 22A-MTS were co-cultivated with PBMCs (E:T = 20:1) and BiUII (10 ng/ml) in the presence (5 μg/ml) or absence of SDH for 24 h. The MTS

Table I. *SDH reduced the release of TNF-α by BiUII -activated PBMCs in co-cultures with MTS. According to FACS data, both cell lines expressed surface EpCAM at comparable levels. It remains unclear why PBMCs consistently released about 3-4 times more TNF-α upon co-cultivation with FaDu MTS. TNF-α values are given in pg/ml.*

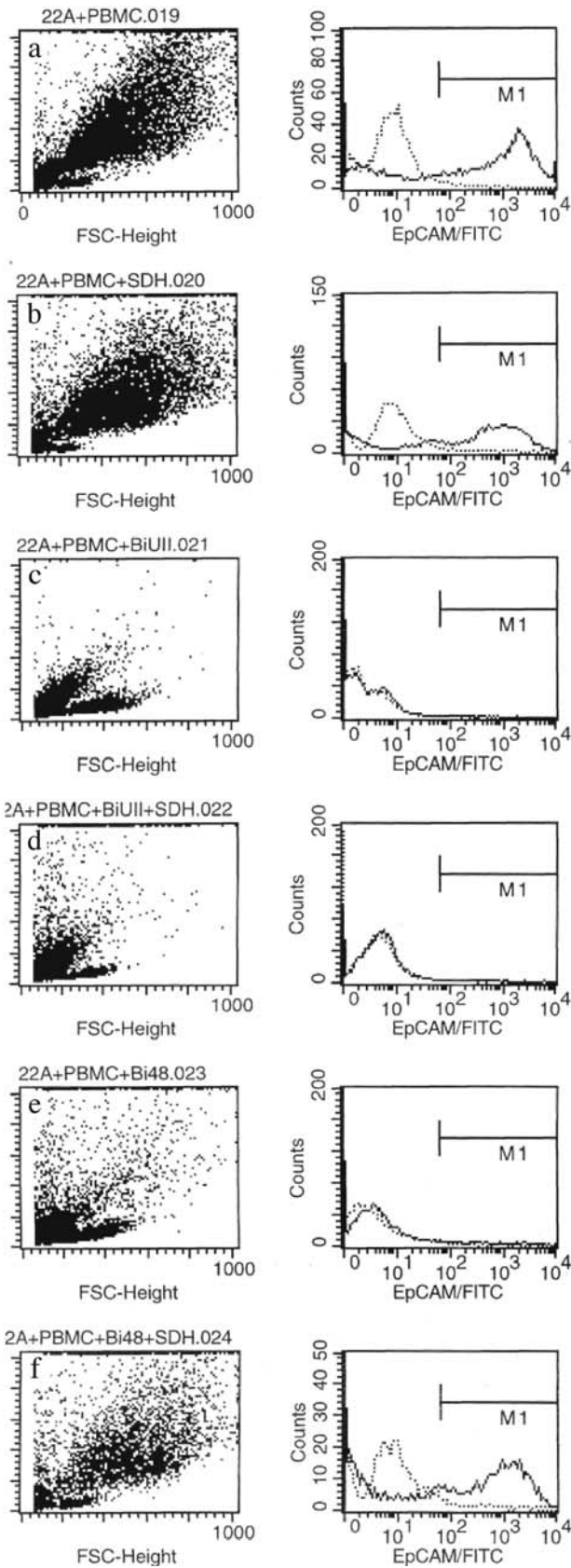| MTS	PBMCs	BiUII	SDH	TNF-α
FaDu				
	–	–	–	13
	+	–	–	367
	+	+	–	9800
	+	+	+	1736
22A				
	–		–	15
	+	–	–	220
	+	+	–	2907
	+	+	+	686

Table II. *Summary of the experiments investigating the influence of SDH in co-cultivations of MTS and trAb-activated PBMCs. PBMCs and MTS were co-cultivated for 24 h, treated with trypsin, and replated in 12-well cluster plates for 3 to 5 days. Residual tumor cells were measured by FACS analysis after staining of cells with an EpCAM-specific antibody. It became clear that SDH did not reduce the activity of BiUII-activated PBMCs, whereas it had an impact on Bi48-induced killing. This discrepancy is most probably due to the absence of the E48 antigen from the tumor cells. Results of one representative experiment out of three are shown.*

MTS	PBMCs	BiUII	Bi48	SDH	Residual tumor cells
FaDu					
	–	–		–	+++
	+	–		–	+++
	+	+		–	–
	+	+		+	–
	+	–	+		–
	+	–	+	+	+
22A					
	–			–	+++
	+	–		–	+++
	+	+		–	–
	+	+		+	–
	+	–	+	–	–
	+	–	+	+	++

were then treated with trypsin to obtain single cell cultures and replated in 6-well cluster plates for 3-5 days. To determine the number of vital tumor cells, the cells were stained with an EpCAM-specific antibody and analyzed by FACS. No EpCAM-positive cells were detectable from co-cultures of MTS and trAb-activated PBMCs, irrespective of whether SDH had been added or not. In contrast, EpCAM-positive cells were easily detectable from co-cultures without trAb (Figure 1a, b). Of interest, the killing of target MTS by Bi48, a similar trAb, the target antigen of which is absent from 22A and FaDu cells, was clearly impaired by SDH (Figure 1e, f).

Discussion

Antibodies, either mono- or bispecific, are promising tools for the immunological elimination of residual tumor cells. However, routine clinical application of trAbs may be complicated or even hindered by serious side-effects, including the release of critical levels of cytokines by activated PBMCs. Consequently, there is great interest in co-medications, which reduce the side-effects without impairing the specific antitumor activity. In order to investigate the effects of prednisolone in this respect, we set up an experimental *in vitro* system consisting of MTS of tumor cell lines, which were co-incubated with allogeneic PBMC and different trAb.

Our previous experiments with this experimental system demonstrated that trAbs induce an efficient antitumor activity in complex three-dimensional tumor structures, as represented *in vitro* by MTS, which are recognized as a valid surrogate system for non-vascularised micrometastases. We also demonstrated that trAb-mediated activation of PBMCs is accompanied by the release of high levels of TNF-α, and that this cytokine contributes to tumor cell killing (12).

Here, we showed that prednisolone significantly reduced the release of TNF-α by BiUII-activated PBMCs. Of interest, this reduction had no negative effect on the killing of EpCAM-expressing tumor cells. Further, it was demonstrated that the killing of target cells is also strictly dependent on the presence of a target antigen, since a similar trAb (Bi48), directed against an irrelevant antigen (E48) induced target cell killing only in the absence of prednisolone. Bi48 provoked the release of similar levels of TNF-α as BiUII, but it may not induce other killing mechanisms such as perforin (10). These results point towards the importance of the "bridging function" of bispecific molecules, which bring together target tumor cells and immune effectors.

Similar results were obtained with a trAb (BiLu) that recognizes tumor cells *via* EpCAM, but binds to the murine instead of the human CD3 molecule on T lymphocytes (data not shown). BiLu led to the release of inferior levels of TNF-α and incomplete killing of the target cells. Since BiLu is of the same isotype composition as BiUII and, therefore, binds to and activates Fcγ-R+ accessory cells like monocytes, this killing is probably attributable to monocytes/macrophages.

In summary, our data provide evidence that trAbs trigger the killing of antigen-positive target cells by bringing together and activating immune effector cells, above all T

Figure 1. *SDH has no influence on the antitumor activity of BiUII-activated PBMCs. 22A-MTS were co-cultivated with PBMCs in the presence (5 μg/ml, b, d, f) or absence of SDH (a, c, e). PBMCs were either activated with BiUII (c, d), with Bi48 (e, f) or were not (a, b). After co-cultivation, the cells were replated and analyzed by FACS for the presence of EpCAM-positive cells. SDH had no influence on the killing of 22A cells by BiUII-activated PBMCs. In contrast, killing by Bi48-activated PBMCs was significantly impaired (f). This difference is most probably due to the absence of the E48 antigen on 22A cells. Measurement of vital tumor cells by FACS was done as described in the Materials and Methods section. 22A and FaDu cells can easily be discriminated from PBMCs with an EpCAM-specific antibody. EpCAM-positive tumor cells are within the marker M1. Dotted lines: isotype control. Similar results were obtained with FaDu-MTS (not shown).*

lymphocytes. The systemic *in vivo* application of trAb may often be limited by overwhelming cytokine concentrations. We demonstrate, here, that the addition of the glucocorticoid prednisolone to co-cultures of MTS and trAb-activated PBMCs resulted in a significant reduction of TNF-α release. In parallel, we were not able to detect a reduced antitumor activity of BiUII-activated PBMCs, at least in this experimental setting. Prednisolone may, thus, be a suitable co-medication, which relieves antibody-induced serious side-effects without impairing the specific killing of target tumor cells.

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