Abstract. The aim of this study was to investigate whether the local induction of pro-inflammatory cytokines in mouse lungs would increase the therapeutic effect of cyclophosphamide (CTX) used to treat experimental B16(F10) melanoma lung metastases. CTX shows anti-angiogenic properties and inhibits the growth of metastases, albeit without numerical reduction. To destroy small metastases remaining after CTX treatment, pro-inflammatory cytokines were induced by systemically administering plasmid DNA-PEI polyplexes. The CpG sequences present in plasmid DNA are immunostimulatory, i.e. they induce pro-inflammatory cytokines, such as IL-12, TNF-α, IFN-γ and IFN-α. The latter has great therapeutic potential as it activates NK cells directly involved in eliminating metastatic foci. Our data indicated, for the first time, that combining cyclophosphamide delivery and local induction of pro-inflammatory cytokines in the lungs with plasmid DNA resulted in reduction in the size of malignant melanoma metastases and their number in mouse lungs. Both effects appeared to contribute to a significant extension of survival.

Despite progress in the treatment of malignant diseases, the low efficacy of therapeutic strategies aimed at preventing the metastatic spread of cancer cells has remained a serious clinical challenge. The formation of secondary metastatic foci represents the final stage of neoplastic disease and it is estimated that 90% of cancer-related deaths are related to metastases (1).

The most limiting and, thus, critical stage of metastatic spread is the terminal one, i.e. the formation of secondary foci (colonies). From approximately 80% of the neoplastic cells undergoing extravasation only 2% establish micrometastases, of which only 0.02% form macro-metastases (2). At this stage of metastatic spread, so-called "dormant" metastases are frequently observed. According to Holmgren et al. (3), metastases remain dormant due to an equilibrium between the proliferative potential of neoplastic cells and their susceptibility to apoptosis, resulting in turn from the balance between pro- and anti-angiogenic factors in the tumor microenvironment. It appears that delivery of an anti-angiogenic agent may shift this balance in the direction of the formation of dormant metastases (3). The anti- and pro-angiogenic balance could be affected, for example, by cyclophosphamide (CTX), a well-known chemotherapeutic agent. According to Browder et al. (4), this compound, when administered in appropriate doses, induced apoptosis of endothelial cells, thereby inhibiting angiogenesis in primary tumors. The anti-angiogenic properties of CTX were also demonstrated by its induction of thrombospondin-1, a potent angiogenesis inhibitor (5).

In order to destroy small metastatic foci remaining after treatment with CTX, local induction of pro-inflammatory cytokines was brought about in the vicinity of metastases by achieving plasmid DNA delivery into the lungs. A number of studies have convincingly demonstrated that systemic delivery of plasmid DNA, using both liposomal and polymeric carriers, such as PEI, results in efficient transfer, primarily to the lungs and to a lesser extent to other organs (see, for example, (6)).

The induction of cytokines by CpG sequences present in plasmid DNA with subsequent cytokine-triggered activation of NK cells at the site of micrometastases (especially by IFN-α) may play a substantial role in inhibiting their growth and eventual elimination (7-9).

Here, it is shown that an appropriate combination of cyclophosphamide delivery and local induction of an inflammatory state indeed decreased the size and number of metastases in mouse lungs and, moreover, resulted in extended survival of the treated animals.
Materials and Methods

Plasmids. The pBCMGNeo plasmid was obtained from Dr. H. Karasuyama (10). Plasmid DNA was isolated and purified using Endo-Free Plasmid Giga Kit columns (Qiagen), as specified by the manufacturer. The amount of endotoxin in the DNA preparations, determined using a Limulus Amebocyte QCL-1000 kit (Bio Whittaker), did not exceed 0.03 EU/μg DNA.

Animals. Six- to eight-week-old C57Bl/6 mice from an on-site animal facility were used in the experiments. The necessary permission for studies involving animals was obtained from the local Ethics Commission (Warsaw Medical University, Warsaw, Poland).

Experimental metastases of murine B16(F10) melanoma. The cells were grown using RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (ICN) in a 5% CO2 atmosphere. The lungs were fixed in Bouin’s solution and visible metastases were determined using a Limulus Amebocyte QCL-1000 kit (Bio Whittaker), did not exceed 0.03 EU/μg DNA.

Preparation of polyplexes. PEI/DNA polyplexes were freshly prepared in polystyrene test tubes (11). Into 13.5 μL of 0.1 M PEI (25 kDa) solution were added 286.5 μL of 5% dextrose, followed by the dropwise addition of 30 μL DNA at 1 μg/μL (in 270 μL of 5% dextrose) and intensive mixing. After a 15-min incubation at room temperature, the emulsion obtained was administered via the animal’s tail vein (final volume 600 μL).

Determination of IFN-α level in lung homogenates. The cytokine levels in the lungs were determined after 3, 6 and 9 h following polyplex administration. The excised lung tissue was placed in 1 mL cold lysis reagent buffer (Promega kit) supplemented with protease inhibitors (benzamidine, PMSF and EDTA (pH 8.0), each at 1 mM final concentration). The lungs were homogenized and the preparation obtained was centrifuged (10 min, 15000 rpm, 4°C). The IFN-α levels in the homogenates were assessed using a commercial kit (R&D Systems).

Hematoxylin and eosin (H&E) staining. The excised lungs were fixed for 24 h in Bouin’s fluid and were then washed with ethyl alcohol (70% for 48 h, 80% for 2 h and 96% overnight). The dehydrated lungs were incubated twice for 1 h in paraffin (58°C) and were then routinely embedded. Sections (8 μm thick) were stained with H&E, as described previously (12).

Statistics. Statistical analysis of the results was performed using the Mann-Whitney U-test (number of lung metastases), whereas the Kaplan-Meier and log-rank tests were applied to assess survival. The results were considered significant at p<0.05.

Results

The influence of CTX alone on the growth of experimental murine B16(F10) melanoma lung metastases was determined first. The most pronounced shrinkage of metastatic foci was observed in animals who had received CTX twice (after 7 and 14 days following inoculation with tumor cells). The third and fourth CTX administrations did not further improve this result. Nevertheless, the CTX-induced size decrease was not accompanied by a numerical reduction of metastases (Figure 1A, C).

Whether the reduced size of the metastases coincided with their decrease in number and prolonged survival when drug delivery was combined with local induction of pro-inflammatory cytokines in the lungs was also investigated. The animals were CTX-treated and injected intravenously with plasmid DNA-containing polyplexes in order to achieve subsequent plasmid DNA release in the lungs. Plasmid DNA is rich in unmethylated CpG sequences, which induce an inflammatory state at the site of plasmid presence via cytokines such as IL-12, TNF-α, INF-γ and INF-α (8, 13).

In our previous study, a 1 μg dose of plasmid DNA did not induce the expression of a related cytokine (14). Here, the INF-α concentration in lung homogenates was determined at 3-, 6- and 9-h time-points following the administration of either 10 μg or 30 μg of plasmid DNA (pBCMGNeo). While those polyplexes containing 10 μg induced an IFN-α level comparable to the control (i.e., ca. 60 pg/ml after 3 h), the transfer of 30 μg raised the concentration of this cytokine to a maximum of 220 pg/ml after 3 h (data not shown).

The effect of combined therapy was investigated using CTX and plasmid DNA. Twice-repeated CTX administration and three doses of plasmid DNA-containing polyplexes (the best combination tested, not shown) in terms of survival extension, diminished the size of the lung metastases and resulted in a concomitant drop in their number from 104±12 in the untreated group or the group treated with CTX alone, to 45±13 in the group treated with both CTX and the pro-inflammatory cytokine-inducing plasmid DNA (Figure 1E). The decreased number of metastases was apparently related to the observed infiltration of immune cells, most probably of NK cells. It should be noted that this infiltration has been seen only in the wake of CTX administration and local inflammation elicited by the administration of 30 μg of plasmid DNA (Figure 1F). In the remaining groups, no such effects could be seen (Figure 1B, D).

A statistically significant survival extension was found for mice treated with the combined therapy (CTX and plasmid DNA) as compared to those mice treated with CTX or plasmid alone (p<0.00018) (Figure 2).

Discussion

CTX is commonly used to inhibit the growth of primary tumors. According to Browder et al. (4), CTX at appropriate doses had an anti-angiogenic effect by inducing the apoptosis of endothelial cells. Additionally, CTX induced thrombospondin-1, a potent angiogenesis inhibitor, which is probably involved in the formation of "dormant" metastases (5). As
seen from our data, CTX did not reduce the number of metastases forming, its effect being limited to retardation of their growth (Figure 1C).

The additional administration of an immunostimulatory agent, such as plasmid DNA containing CpG sequences, activated cells of the immune system, mainly NK cells, which might lead to the elimination of a number of small metastases (7). It was noticed that the induced inflammatory state neither retarded growth nor reduced the number of large metastases; it only eliminated small "dormant" metastases which appeared following CTX treatment. The decrease in number of the small "dormant" metastases brought, as a result, extended survival of the treated mice (Figure 2). A marked, statistically significant improvement in survival, compared to the controls (plasmid DNA or CTX alone), was seen only in animals treated with a combination of CTX and the induced inflammatory state (via administration of complexed plasmid DNA) (Figure 2). The therapeutic effect was dependent on two factors, namely a local induction of the inflammatory state and an appropriate combination of CTX and locally-activated pro-inflammatory cytokines.

The main finding of the study was that only an appropriate combination (two doses of CTX and three doses of polyplexes) of both therapeutic modalities studied was effective in reducing both the size and number of metastatic foci. This might mean that relationships between induced cytokines (which, in addition to inducing NK cells, also induce pro-angiogenic VEGF (15)) on the one hand, and cyclophosphamide inducing thrombospondin-1 on the other, are of particular significance and need to be investigated in-depth.
Our results demonstrated, for the first time, an augmentation of the therapeutic effect of cyclophosphamide via local induction of pro-inflammatory cytokines at the site of metastases.

Acknowledgements

This study was supported by a Grant from the State Committee for Scientific Research (KBN) No. 3PO5A04422, Poland.

References


Received November 16, 2005
Revised February 2, 2006
Accepted March 22, 2006