Short Communication

Immunotherapy of HPV 16-Associated Tumours with Tumour Cell Line/Dendritic Cell Line (TC-1/DC2.4) Hybrid Vaccines

(HPV16 / dendritic cells / fusion)

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Abstract. Hybridization of established dendritic cell lines with tumour cells represents a prospective technology for the construction of antitumour vaccines. Experiments were designed to examine whether administration of cell populations prepared by fusion of HPV 16-associated tumour TC-1 cells with dendritic cell line DC2.4 could be used for treatment of TC-1 tumours growing in syngeneic mice. The therapeutic potency of TC-1/DC2.4 fusion vaccine administered 24 h after fusion and that of TC-1/DC2.4 hybrid cells selected for 3 weeks in HAT-containing medium was tested. It has been found that administration of both types of fusion vaccines at the site of growing TC-1 tumour transplants significantly inhibited tumour growth with regard to the percentage of tumour-bearing mice and to the size of the transplanted tumours. Peritumoral administration of the DC2.4 cells alone also reduced the size of growing TC-1 tumours, but not the percentage of the tumour-bearing mice. Although in the groups of mice treated with fusion vaccines the size of the tumours was reproducibly smaller than that in the mice treated with parental DC2.4 cells, the difference was not statistically significant.

Dendritic cells (DC) are professional antigen-presenting cells capable of inducing potent antitumour immunity (Steinman, 1991). Various strategies have been developed for utilization of DC as tumour vaccines. These strategies were based on the loading of DC with tumour-associated oncoproteins, with oncoprotein-derived synthetic peptides, with DNA or RNA coding for tumour-associated antigens, or on the utilization of tumour cell/DC hybrids (for a review, see Bubeník, 2001; Reinhard et al., 2002). The advantage of tumour cell/DC hybrids is provided by the expected simultaneous expression of various tumour-associated antigens in the hybrids and by their presentation in the context of high levels of MHC class I, MHC class II and costimulatory molecules of the DC partner. This approach also allows for the development of antitumour immunity directed against uncharacterized tumour antigens and their multiple epitopes.

Murine DC fused with tumour cells induced antitumour immunity in several tumour systems (Gong et al., 1997; Wang et al., 1998; Oretas et al., 2001; Chen et al., 2003). Utilization of DC lines for these purposes can provide a standard, well-characterized and rapidly accessible material. Our previous findings have shown that the established dendritic cell line DC2.4 could be utilized successfully for therapy of HPV16-associated MK16/1/IIIABC tumours (Mendoza et al., 2003). In this paper we have used the DC2.4 cell line for treatment of another HPV 16-associated neoplasm, TC-1, and for the construction of hybrid TC-1/DC2.4 cell population for vaccination purposes.

Material and Methods

Mice

C57BL/6 males, 2–4 months old, were obtained from AnLab Co., Prague, Czech Republic.

Cell lines

The DC2.4 cell line was established from bone marrow cells of C57BL/6 mice after infection with a retrovirus encoding v-myc and v-raf (Shen et al., 1997) and was generously provided by Dr. K. L. Rock (Department of Pathology, University of Massachusetts Medical School, MA). The TC-1 cell line was prepared by transformation of C57BL/6 primary mouse lung cells with HPV 16 E6/E7 oncogenes and activated H-*ras* (Lin et al., 1996). The TC-1 cell line was maintained in RPMI 1640 medium supplemented with

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Abbreviations: DC – dendritic cells, HAT – hypoxanthine aminopterin deoxythymidine, HPV – human papilloma virus, MHC – major histocompatibility complex.

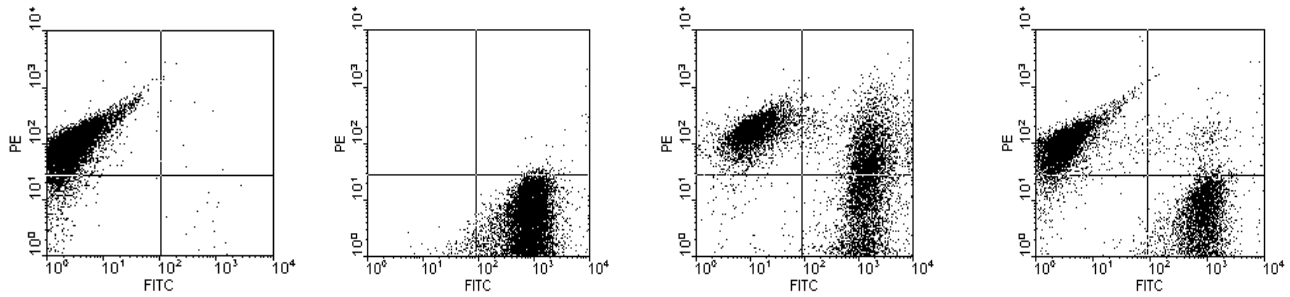


Fig. 1. Cytofluorometric analysis of hybrid TC-1/DC2.4 cell populations. DC2.4 dendritic cells and TC-1 cells were stained red or green, respectively, using PKH26 and PKH67 linkers (Sigma). After fusion, the cells were suspended in RPMI 1640 medium and subjected to cytofluorometric analysis. (a) DC2.4 cells, (b) TC-1 cells, (c) 24 h TC-1/DC2.4 cells, (d) mixture of TC-1 + DC2.4 cells.

10% foetal calf serum (BioClot Ltd., Aidenbach, Switzerland), 2 mM L-glutamin, penicillin and streptomycin; in the case of DC2.4, additional 2 x 10^{-5} M 2-mercaptoethanol was added.

Cell fusion

Construction of 24 h TC-1/DC2.4 vaccines. Briefly, DC2.4 cells were mixed with the TC-1 cells at the 6 : 1 ratio and washed in serum-free RPMI 1640 medium. Polyethylene glycol dimethylsuphoxide solution (50% PEG/10% DMSO in PBS, Sigma, Steinheim, Germany) was added by drops to the cell mixture. After dilution with serum-free RPMI 1640 medium, the cells were centrifuged, suspended in the complete RPMI 1640 medium and incubated overnight at 37°C in 5% CO_2. After 24 h the cells were irradiated with 100 Gy and used for the therapy. To determine the efficiency of fusion, DC2.4 and tumour cells TC-1 were stained red or green, respectively, before fusion using PKH26 and PKH67 linker kits (Sigma) according to the manufacturer's directions (Kikuchi et al., 2001). Flow cytometry analysis of a typical fusion between DC2.4 and TC-1 cells is shown in Fig. 1. The results showed that 92.3% of DC2.4 (Fig. 1a) and 97.1% of TC-1 (Fig. 1b) cells were stained prior to the fusion. Two hours after fusion, 23.6% of dual-coloured cells were found (Fig. 1c). In the mixture of DC2.4 and TC-1 without fusion, 3.1% dual coloured-cells (Fig. 1d) were detected. With this method, however, we could not distinguish between cell hybrids and cell clusters (Celluzzi and Falo, 1998).

Construction of TC-1/DC2.4 hybrid vaccines followed by selection in the HAT-containing medium. DC2.4 cells 6-thioguanine-resistant and defective for hypoxanthine-guanine phosphoribosyl transferase (Šímová, unpublished data) were mixed with 5'bromodeoxyuridine-resistant TC-1 cells defective for thymidine kinase (Šímová, unpublished data) at the 1 : 1 ratio. Cells were washed in serum-free RPMI 1640 medium and fused using polyethylene glycol dimethylsulphoxide solution (50% PEG/10% DMSO in PBS, Sigma) and stirring. Additional serum-free RPMI 1640 medium was added slowly, cells were centrifuged and resuspended in the RPMI 1640 medium containing HAT (medium supplemented with 10^{-4} M hypoxanthine, 3.8 x 10^{-7} M aminopterin and 10^{-5} M 2-deoxythymidine, Sigma). After three weeks of cultivation in the selection medium the hybrid cells were irradiated with 100 Gy and used for the therapy.

Immunotherapy

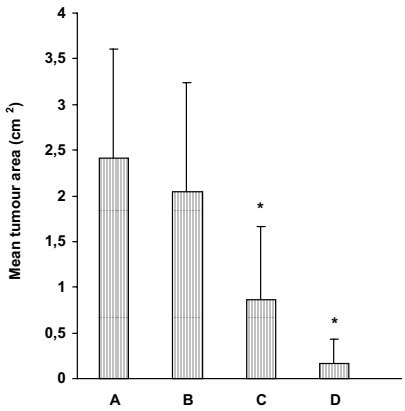
C57BL/6 mice were inoculated s.c. with 5 x 10^4 TC-1 cells on day 0. Four days after challenge the mice were vaccinated with 100 Gy-irradiated 3 x 10^6 fused cells. The tumour-bearing mice were observed twice a week and the numbers of tumour-bearing mice and size of the tumours were recorded. For statistical analysis of the differences between the size of the tumours, the analysis of variance from NCSS, Number Cruncher Statistical System statistical package, was used. For comparison of tumour takes in experimental and control groups, the χ^2 comparison test was utilized.

Results and Discussion

Therapeutic efficacy of 24 h TC-1/DC2.4 vaccines

Mice were s.c. inoculated on day 0 with 5 x 10^4 TC-1 cells and on day 4 the mice were s.c. injected in the vicinity of the tumour transplants with irradiated TC-1/DC2.4 fusion vaccines. As a control, irradiated TC-1 cells or irradiated DC2.4 cells were used. As can be seen in Fig. 2a, administration of irradiated DC2.4 cells, as well as administration of irradiated DC2.4 cells fused with the TC-1 cells inhibited TC-1 tumour growth, as compared to the untreated controls or to the mice treated with irradiated TC-1 cells ($P < 0.01$). The difference between the group of mice treated with DC2.4 cells and the TC-1/DC2.4 vaccine was not significant. As shown in Fig. 2b, administration of the TC-1/DC2.4 fusion vaccine significantly reduced the percentage of tumour-bearing mice as compared to untreated controls or to the mice treated with irradiated TC-1 cells ($P < 0.025$). In contrast, administration of

a

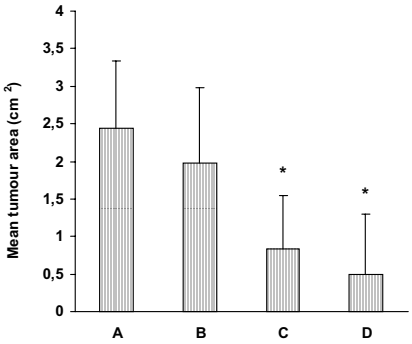


b

Group of mice	No. of mice with tumour / total No. of mice	P <
Untreated (A)	10/10	
TC-1 cells (B)	9/9	
DC2.4 cells (C)	6/8	
DC2.4 × TC-1 fusion vaccine (D)	4/8	0.025**

Fig. 2. Inhibitory effects on 5-day s.c. TC-1 tumours of TC-1/DC2.4 vaccine, 24 h after fusion. The experimental mice were inoculated with TC-1 on day 0, and 4 days after challenge were injected with the respective vaccine. The experiment was terminated and evaluated on day 46. (a) *P < 0.01 as compared to untreated controls or TC-1 cells. (b) **As compared to untreated controls or TC-1 cells. The experiment was repeated with similar results.

a



b

Group of mice	No. of mice with tumour / total No. of mice	P <
Untreated (A)	10/10	
TC-1 (B)	8/8	
DC2.4 (C)	7/8	
DC2.4 × TC-1 (D)	3/6	0.025**

Fig. 3. Inhibitory effects on 5-day s.c. TC-1 tumours of TC-1/DC2.4 vaccine after 3-week selection of hybrid cells in HAT-containing medium. The experimental mice were inoculated with TC-1 on day 0, and 4 days after challenge were injected with the respective vaccine. The experiment was terminated and evaluated on day 42. (a) *P < 0.01 as compared to untreated controls or TC-1 cells. (b) **As compared to untreated controls or TC-1 cells. The experiment was repeated with similar results.

irradiated DC2.4 cells did not significantly reduce the percentage of tumour-bearing mice, as compared to untreated controls or TC-1-vaccinated mice.

Therapeutic efficacy of TC-1/DC2.4 fusion vaccine used after 3-week selection of hybrid cells in HAT-containing medium

Mice were s.c. inoculated on day 0 with 5 x 10^4 TC-1 cells. On day 4 the mice were s.c. injected in the vicinity of tumour transplants with irradiated TC-1/DC2.4 hybrid cells, selected for 3 weeks in the HAT-containing medium, and then irradiated with 100 Gy. As controls, untreated mice and mice treated with irradiated TC-1 cells or irradiated DC2.4 cells were used. As can be seen in Fig. 3a, administration of irradiated DC2.4 cells, as well as administration of irradiated DC2.4 cells fused with the TC-1 cells inhibited TC-1 tumour growth, as compared to the untreated controls or to the mice treated with irradiated TC-1 cells ($P < 0.01$). The difference between the group of mice treated with DC2.4 cells and TC-1/DC2.4 vaccine was not significant. As shown in Fig. 3b, administration of the TC-1/DC2.4 fusion vaccine significantly reduced the percentage of tumour-bearing mice as compared to untreated controls or to the mice treated with irradiated TC-1 cells ($P < 0.025$). In contrast, administration of irradiated DC2.4 cells did not significantly reduce the percentage of tumour-bearing mice, as compared to untreated controls or to the TC-1-vaccinated mice.

Efficient antigen presentation is one of the essential components in the development of antitumour immune responses. It has been demonstrated that the number of dendritic cells in the local tumour environment correlates with patients' survival in numerous tumour systems (Dallal et al., 2002). For instance, in gastric cancer, the density of DC served as a useful indicator in evaluation of patients' prognosis; it has been found that the degree of DC infiltration gradually decreased during tumour progression (Ishigami et al., 2000; Coventry et al., 2003). Similarly, infiltration with DC can prevent peritoneal recurrences in patients with advanced gastric cancer (Tsujitani et al., 1992). We have previously found that local increase in the number of bone marrow-derived DC at the site of HPV16-associated MHC class I^- tumour MK16/1/IIIABC inhibited progression of the tumour in syngeneic mice (Mendoza et al., 2002). We have also found that administration of cells of immortalized DC line DC2.4 in the vicinity of MK16/1/IIIABC tumour transplants inhibited growth of these tumours (Mendoza et al., 2003).

In this paper, we have examined the therapeutic potency of DC2.4 cells in another HPV16-associated, MHC class I^+ tumour, TC-1, transplanted in syngeneic mice, and the therapeutic effect of TC-1/DC2.4 hybrid cell vaccines. The reported results indicate that the cells of established dendritic cell line DC2.4 alone can inhib-

it TC-1 tumour growth. The TC-1 tumour cell/dendritic cell line DC2.4 hybrid vaccine was more efficient in the TC-1 tumour immunotherapy than the DC2.4 cells alone. This difference was expressed in the percentage of tumour-bearing mice and less pronounced (non-significant) in the difference of the tumour size. The advantage of utilization of DC lines for the construction of tumour vaccines is given by the fact that the cells of DC lines can be supplied in the required high quantities and that they represent standard, well-characterized cell populations allowing repetition of the experiments under identical conditions. In accordance with our results obtained with the TC-1/DC2.4 hybrid cell vaccine, other groups using hybrid bone marrow-derived DC/tumour cell vaccines have previously demonstrated that the fusion vaccines could also be utilized either shortly after fusion (Kugler et al., 2000; Trefzer er al., 2000), or after selection in HAT-containing medium (Gong et al., 1997).

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