Short Communication

Reduction of 1-Methyl-1-Nitrosourea-Induced Tumor Burden with DNA Vaccines Encoding Mutated Ras Epitopes and the Costimulatory Molecule B7.1

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Abstract. 1-methyl-1-nitrosourea (MNU), a well characterized carcinogen, was used to induce adenocarcinomas in rat mammary gland. 150 days after the first injection of MNU, the animals were treated with DNA minigene vaccines encoding ras T cell epitopes together with the co-stimulatory molecule B7.1 (CD 80). Five injections with a biolistic device (gene gun) in monthly intervals significantly reduced the tumor burden. A therapeutic effect could be measured with both, DNA vaccines encoding ras epitopes and B7.1, as well as with a DNA vaccine expressing solely the B7.1 molecule thus indicating the potential of genetic vaccination for tumor treatment.

Key words: Tumor — MNU — Genetic immunization — DNA vaccine — DNA minigene — H-ras

Abbreviations: MNU, 1-methyl-1-nitrosourea; Ag, antigen; CTL, cytotoxic lymphocyte; MCS, multiple cloning site; pCMV, plasmid with cytomegalovirus promotor; TPA, tissue plasminogen activator; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; ODN, oligodeoxynucleotide; DC, dendritic cell.

MNU is a well characterized carcinogen that induces adenocarcinomas in rat mammary gland with high specificity. This model has been proven to be of resemblance to human breast cancer and is therefore of great interest for tumor studies (Mehta 2000). The mechanism of tumor induction and progression is still not fully understood. Transition of Gly → Glu at codon 12 of H-ras, occurring in 60–80 % of tumors represents the best characterized mutation. However, at present its signifi-

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cance in adenocarcinomas in rat mammary gland is controversially discussed (Cha et al. 1994; Lu et al. 1998).

Nevertheless, ras mutations belong to the most prominent tumor inducers and are attractive targets for tumor treatment. We have recently developed DNA minigene vaccines encoding T cell epitopes of the murine H-ras (covering the amino acids 4–12 and 4–16, respectively) with a mutation at codon 12 (Gly → Val) and investigated whether they could induce Ag-specific $CD8^+$ cytotoxic and $CD4^+$ lymphoproliferative responses. In addition, we compared two different immunization procedures, epidermal gene gun inoculation and intradermal injection of saline plasmid DNA, along with several approaches addressing different aspects of immune modulation. The results demonstrated that each DNA plasmid induced the relevant Ag-specific cellular immune response and that gene gun inoculation was superior to that of needle injection. Moreover, the study also elicited that a DNA plasmid expressing nested mutant ras epitope-specific $CD4^+$ and $CD8^+$ T cell epitopes can be processed *in vivo* to induce both subset-specific T cell responses, and that the addition of the helper epitope quantitatively improved the development of the CTL response (Bristol et al. 2000; Lindinger et al. 2003). Furthermore, this approach also demonstrated the possibility to use DNA minigene vaccines for inducing a specific immune response against single amino acid exchanges in mutated proteins. Based on these data, the present study was aimed at testing the therapeutic effect of minigene vaccines against H-ras mutation at codon 12 *in vivo* with a MNU-induced rat mammary gland adenocarcinoma model system.

Five groups of female Sprague-Dawley rats received MNU to induce mammary gland adenocarcinoma at day 50, 219 and 274, with doses of 50, 25 and 25 mg/kg bodyweight, respectively.

For the treatment three different vaccines were employed: (1) a DNA minigene encoding the T-helper epitope containing the amino acids 4–16 with the mutation Gly → Glu at position 12 (designated pCMVras4–16); (2) the same DNA minigene with an additional sequence encoding a tetanus toxin helper epitope (pCMVras4–16/t); (3) a DNA vaccine with the H-ras cDNA coding sequence of the amino acids 2–60, again containing the mutation Gly → Glu at position 12 (pCMVras2–60).

All constructs were cloned into the multiple cloning site (MCS) of the pCI (Promega) vector backbone following a TPA leader sequence to enhance transport of the translated peptides into the endoplasmatic reticulum (Weiss et al. 1999).

We have recently demonstrated the benefitial effect of co-application of constructs encoding immunostimulatory molecules on the induction of cytotoxic T cells with mutated murine H-ras in the mouse (Lindinger et al. 2003). Therefore, in the present study a second construct carrying the murine B7.1 (CD 80) cDNA coding sequence in the MCS of pCMV (pCMVB7) was used as adjuvant for all experimental groups.

The approach was performed with five different groups: (1) pCMVras4–16 + pCMVB7, (2) pCMVras4–16/t + pCMVB7, (3) pCMVras2–60 + pCMVB7, (4) pCMV (empty vector) + pCMVB7 and (5) a MNU-injected control group

Table 1. Tumor evaluation of experimental and control groups

	Tumors per animal	Tumor weight [g]	Tumor volume [mm^3]
pCMVras4–16+pCMVB7	1.50 (0.00–3.00)	*0.97 (0.00–2.52)	*960.50 (0.00–4488.00)
pCMVras4–16/t+pCMVB7	1.50 (0.00–4.00)	*0.64 (0.00–11.83)	*990.00 (0.00–23,328.00)
pCMVras2–60+pCMVB7	2.00 (0.00–3.00)	3.26 (0.00–30.26)	9205.00 (0.00–77,056.00)
pCMV+pCMVB7	*0.00 (0.00–4.00)	0.00 (0.00–221.00)	0.00 (0.00–291,040.00)
control	3.00 (1.00–7.00)	6.02 (0.75–30.77)	8542.00 (1330.00–62,402.00)

Values are given as median (5–95 % range), values differing significantly from control group are indicated by asterisks.

without vaccine treatment. Both plasmids of each group were coated onto gold beads (diameter 1.6 μm) and delivered into the epidermis of rat skin by the use of a gene gun at a pressure of 400 psi. The vaccine was applied at two non-overlapping sites (each injected with 0.5 mg gold coated with 2 μg plasmid DNA) on days 151, 179, 207, 235 and 274. Animals were sacrificed 5 weeks later (day 308) and the tumor burden was evaluated in terms of tumor number *per* animal, as well as weight and volume of each tumor (Table 1).

The groups treated with pCMVras4–16 and pCMVras4–16/t showed a significant decrease of tumor burden compared to the untreated control group, while the group receiving the vaccine encoding a longer fragment of ras (pCMVras2–60) showed a strong tendency to lower tumor burden, but didn't reach significance in our experiment. Surprisingly, injection of a gene vaccine containing a mock vector together with the co-stimulatory molecule B7.1 elicited a significant reduction of tumor burden as well. This finding indicates that either expression of B7.1 or the gene gun bombardment itself induced an immunostimulatory effect which apparently triggered and/or enhanced the natural anti-tumor response. Another explanation for this unexpected effect could be the activation of the immune system *via* immunostimulatory DNA motifs, so-called CpG motifs (Leitner et al. 2001). CpG motifs are immunomodulatory DNA motifs that represent a strong "danger" signal for the innate immune system and act as a Th1-type adjuvant *via* the induction of type-1 interferons such as IFN-α/β, IL-1β, TNF-α, IL-6 and IL-12 (Krieg 2002). We have recently shown a markedly reduction of tumor burden of MNU-induced mammary gland adenocarcinoma in female rats after treatment with oligodeoxynucleotides containing CpG (CpG-ODN) motifs (Macejova et al. 2001). However, with respect to the present study, it can be assumed that the amount of 2 μg DNA, bearing only a few CpG sequences, is not sufficient to trigger a systemic "danger" signal comparable to that of 100 μg of pure CpG-ODN as used for the study cited above.

Therefore, an explanation of the therapeutic efficacy of the pCMV + pCMVB7 DNA vaccine must focus on the effect of the B7.1 molecule and/or the effect of gene gun bombardment itself. The former belongs to a group of costimulatory

molecules usually expressed on professional antigen presenting cells to provide the necessary second signal of activation to interacting T cells (Bueler and Mulligan 1996; Horspool et al. 1998). The co-delivery of B7.1 together with a vaccine may lead to stronger T cell induction and proliferation and therefore to the enhanced recruitment of effector cells (Kim et al. 1998; Lindinger et al. 2003). In the case of co-delivery of B7.1 with an empty vector, the increased expression of B7.1 on epidermal and dermal cells and on migrated and activated dendritic cell types in the lymph nodes may be sufficient to enhance the immunity against tumor material provided by draining the tumor area.

Gene gun bombardment is known to trigger migration and activation of dendritic cells (DC) in the dermis and of Langerhans cells in the epidermis (Porgador et al. 1998) and represents a particular "danger" signal even without plasmid DNA (Weiss et al. 2002). Gene gun treatment results in a more than 200 fold excess of activated DC *versus* transfected DC, independent of the presence of plasmid DNA. In combination, the effects of the increased B7.1 expression and that of the gene gun bombardment fulfill the theoretical criteria for triggering an effective anti-tumor immune response *in vivo*.

Summing up, the present data cleary demonstrate a therapeutic effect of genetic immunization with DNA constructs encoding epitopes of the ras oncogene and the costimulatory molecule B7.1. The results also indicate that solely the stimulation of the innate immune system by enhanced expression of costimuli *via* DNA vaccines seems to be sufficient to lower the tumor burden. Further studies must try to shed light on the role of these processes.

Acknowledgements. This work has been supported by the Centre of Excellence grant ICA1-CT-2000–70008 and in part by the VEGA grant No. 2/2070/22 and the Austrian Krebshilfe Salzburg.

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Final version accepted: December 4, 2003