Liver Carcinogenesis

The Molecular Pathways

Edited by

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THE GENERATION OF TUMOR VACCINES BY ADENOVIRUS-ENHANCED TRANSFERRINFECTION OF CYTOKINE GENES INTO TUMOR CELLS

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INTRODUCTION

The major obstacle to immunotherapy of cancer is the poor immunogenicity of tumors, especially in man. This is somewhat surprising since the large number of genetic alternations found in advanced cancers should give rise to peptide neo-epitopes capable of being recognized in the context of MHC-I molecules by cytotoxic lymphocytes (Lurquin et al., 1989). Indeed, tumor-associated (Groen et al., 1987) and tumor-specific antigens (van der Bruggen and van den Eynde, 1992) which should be targets for immunological attack have recently been identified. This leads to the suspicion that foreign antigens are indeed present on most, or at least many, tumor cells and that tumor cells are not rejected as foreign because the response of the immune system to the presented foreign antigen is inadequate (Fearon et al., 1990).

If tumor-specific antigens can be identified on all or most kinds of tumors, it is conceivable that someday antibodies against the tumor antigens may be used to combat cancer of that recombinant antigens can be used to elicit

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cellular immunity against tumors. Since to date the distribution and nature of tumor antigens is mainly unknown, attempts are being made to use the entire tumor cells, which were transfected with cytokine-expressing plasmids as a source of antigen and to rely on the immune system to seek out and to mount a response against such foreign antigens.

It was found empirically (Fearon et al., 1990) that immunological tolerance of tumors can be broken by transfecting tumor cells with IL-2-gene expression vectors. When such cells are transplanted into syngeneic mice, a powerful systemic response based on T-lymphocytes is mounted by the organism leading to the destruction of both the IL-2 expressing tumor cells as well as parental tumor cells (not expressing cytokines) injected at distant sites. The systemic response does not derive from an increased systemic level of IL-2 as a consequence of implantation of cytokine producing tumor cells, rather it is the high level of cytokines arising locally, which is thought to have a dramatic effect on reprogramming the immune system.

This initial observation has led to an avalanche of rather unsystematic studies for many mouse tumor models using different cytokine genes (reviewed by Zatloukal et al., 1993) in which rejection of parental tumor cells was reproduced using the above mentioned strategy. It is hoped that such findings will ultimately lead to clinical protocols in which the primary tumor will be removed from patients, the tumor cells set to culture, transfected with cytokine expression vectors, inactivated with X-rays and implanted as tumor vaccines back into patient. If a strong immune response results, one can be hopeful that this procedure will lead to an eradication of distant micrometastases over a long time which have arisen previously from disseminated tumor cells.

Transferrinfection of Tumor Cells for the Generation of Tumor Vaccines

Since the tumor tissue may be heterogeneous due to tumor progression and selection by the host immune system, it may be desirable not to use transfected or transduced cell clones, as has been done in most experiments reported up to now (Zatloukal et al., 1993). This procedure, besides being time consuming and delaying unnecessarily the application of the "tumor vaccine" may inadvertently lead to selection of an unrepresentative cell clone. Furthermore, extensive culturing and expansion of cells may lead to a loss of the tumor antigen. A procedure by which the bulk of the tumor cells can be transfected soon after removal and culture would seem desirable.

We believe that our recently developed adenovirus-augmented, receptor-mediated DNA transfer technique "Adenovirus-Enhanced Transferrinfection" (AVET) (Cotten et al., 1992, Curiel et al., 1992, Wagner et al., 1992, Zatloukal et al., 1992) has many advantages over retroviral transduction or DNA transfection followed by clonal expansion of the genetically modified cells. AVE is a new transfection protocol in which the plasmid DNA to be transported into the tumor cells is reacted with transferrin-polylysine to form highly condensed round particles with a diameter of approx. 100 nm, referred to as "donuts" (Zatloukal et al., 1992). These "donuts" are linked to adenovirus Ad5d11014 which, owing to its endosomolytic property, greatly enhances the receptor-mediated transfer of genes into cells and ultimately into the cell nucleus (Cotten et al., 1992, Curiel et al., 1992, Wagner et all, 1992, Zatloukal et al., 1992).

One of the outstanding features of AVET is that with this technique a multiplicity of DNA plasmids (per cell) can be introduced into a large fraction

of cells both from cell lines and primary cell culture, including freshly prepared murine and human melanoma cells. Followed transferrinfection, extraordinarily high levels of cytokine or reporter gene expression can be achieved routinely in freshly isolated tumor cells. Cytokine-expressing cells can then be easily mixed with non-transfected irradiated cells to obtain any desired cytokine-expressing level. This seems to be a good starting point for the generation of a tumor vaccine by the procedure described above.

To test the concept of a tumor vaccine, we chose a murine melanoma skin cancer model. For several reasons, melanoma cancer seems to be a particularly interesting target: There is ample evidence that melanomas are subject to immunological control in humans. For instance, there is a (transient) regression of skin cancers in about 25% of the patients and complete remission in about 0.5%, and tumor-specific antigens (MAGE 1, MAGE 3) have been identified (van der Bruggen and van den Eynde, 1992; Gaugler et al., 1994). In addition, cytotoxic T-lymphocytes directed against the tumor can be obtained from patients (Herin et al., 1987; Topallan et al., 1989).

Immunization with IL-2 Transfected Melanoma Cells Protects Mice from Tumor Development

The murine melanoma cell line Cloudman S91 (clone M3) was obtained from ATCC (No. CCL53.1). M3 cells which were established from a spontaneously developed melanoma in DBA/2 mice (Cloudman, 1941) express low level of MHC-I antigens (unpublished observation) and are only moderately immunogenic.

Groups of 6 mice were immunized with IL-2 transfected (24,000 Units/10^6 cells / 24 h), or non transfected tumor cells. Prior to immunization, cells were

irradiated (2,000 rad) to avoid further cell proliferation. Mice were immunized twice subcutaneously with 1 x 10^5 cells into the left flank in a weekly interval. After an additional week, animals were challenged with 1 x 10^5 viable M3 melanoma (30-fold tumorigenic dose) cells into the right flank and tumor growth was scored weekly.

Control animals which only received a challenge dose developed tumors within 2 weeks, whereas M3/IL-2 vaccinated mice were completely protected (fig. 1). 5 out of 6 animals which were immunized with non transfected melanoma cells developed tumors. Our results show that irradiation of tumor cells led to a somewhat slower tumor growth, but was not sufficient for the generation of an adequate immune response in our model.

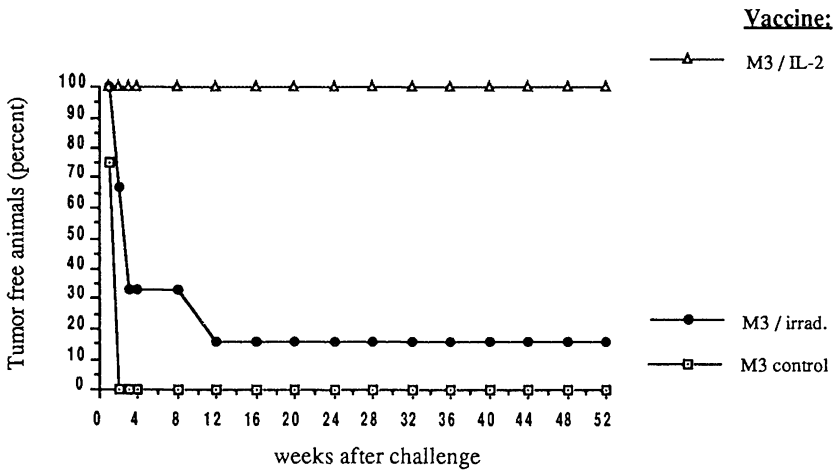

Fig. 1. Groups of DBA/2 mice (each 6 animals) were vaccinated twice in a one week interval with 1 x 10^5 IL-2 transfected, irradiated M3 cells and challenged with 1 x 10^5 viable M3 cells one week later.

AVET leads to very high cytokine expression levels with IL-2 Production rates in M3 cells in vitro of more than 60.000 Units/10^6 cells / 24 h. Since other groups used much lower cytokine levels for vaccination of animals, we were interested whether the high IL-2 production provides any advantage for vaccine efficiency. Vaccines which produced different amounts of IL-2 were generated by using transfection complexes in which the IL-2 expression vector (pWS2m) was mixed with plasmid missing the IL-2 coding region (pSP) in different ratios. This procedure allowed to adjust the IL-2 expression to 400 Units/ 10^6 cells/ 24 h without changing the complex formation.

Table 1. IL-2-dose dependent protection of immunized mice

Challenge/ IL-2-production	1 w	2 w	3 w	4 w	5 w	6 w	8 w
1 x 10^5 M3 IL-2 high	0/6	0/6	0/6	0/6	0/6	0/6	0/6
1 x 10^5 M IL-2 low	0/5	0/5	1/5	1/5	1/5	1/5	1/5
3 x 10^5 M3 IL-2 high	0/8	0/8	1/8	1/8	1/8	1/8	1/8
3 x 10^5 M3 IL-2 low	0/8	1/8	5/8	5/8	5/8	5/8	5/8

Table 1: Mice were immunized twice with 1 x 10^5 IL-2 transfected, irradiated (2000 rad) M3 cells producing either 20.000-30.000 Units IL-2/ 10^6 cells / 24 h (IL-2 high) or 400-500 Units IL-2/ 10^6 cells / 24 h (IL-2 low). One week after the second immunization, the mice were challenged with 1 x 10^5 or 3 x 10^5 parental (tumorigenic) M3 cells. Numbers represent tumor bearing animals/total number of animals at the indicated periods after challenge.

Using this approach, mice were immunized twice with high and low level IL-2 producing vaccines as described above. One week after the second immunization animals were challenged with either 1 x 10^5 or 3 x 10^5 wild-type M3 cells.

We find that there is a dose-dependent relationship between IL-2 expression in vitro and vaccine efficiency in vivo. The low level IL-2 vaccine is less effective than the high level IL-2 vaccine: a lower cancer cell numbers are rejected, tumor development occurs earlier, and fewer animals show protection (Table 1).

Fates of Cytokine-Transfected Tumor Cells In Vivo

The Polymerase Chain Reaction (PCR) amplification technique with an appropriate internal standard for quantitative evaluation was adopted to determine the survival time of IL-2 plasmid and adenovirus DNA (another component of the transfection complex) of subcutaneously injected, transferrinfected and irradiated M3 cells. The persistence of the DNAs was taken as a measure for the survival of the cells at the vaccination site. Three groups of DBA/2 mice (two animals each) were injected with 3 x 10^5 IL-2 transfected, irradiated M3 cells. PCR analysis of the samples taken at day one, two and five after immunization shows a fast destruction of the IL-2 plasmid after subcutaneous injection into the back of the mouse. While still detectable after 24 h and 48 h, the IL-2 plasmid could only be amplified from one DNA sample prepared from skin specimens than had been injected 5 days previously, the second DNA sample from day 5 was negative for IL-2. Adenovirus DNA could be successfully amplified from day 1 and day 2. However, both DNA samples from day 5 were completely negative (Figure 2).

The possible transfer of recombinant or viral DNAs to nearby lymph nodes, to different organs of the animals as well as monocytes and macrophages from the peripheral blood was investigated at high sensitivity. 12 male and 12 female DBA/2 mice were injected with 1 x 10^6 IL-2 transfected M3 cells. 24 h and 48 h after injection, DNA from six animals of each group was prepared from peripheral blood cells and several organs. PCR analysis fails to disclose amplifiable IL-2 adenovirus DNA in all DNA samples from the various mouse organs including draining lymph nodes, spleen, kidney, lung, liver, colon, testis, ovaries, and the peripheral blood mononuclear cells.

In order to determine systemic IL-2 levels following immunization, groups of 3 mice were injected with 1 x 10^6, 3 x10^5 and 1 x 10^5 IL-2 transfected M3 cells which produced 46.000 Units IL-2/10^6 cells/24h in vitro. After 24 and 48 h blood of animals was collected and IL-2 levels in sera were determined by ELISA (Genzyme, Cambridge, MA). As a result, after 24 h systemic levels became detectable in all three mice receiving 1 x 10^6 cells, and in 1 out of 3 mice receiving 3 x 10^5 cells. Animals treated with 1 x 10^5 IL-2 transfected cells, the dose which generates systemic protection, showed no systemic IL-2 levels in the sera. At 48 h after cell implantation, systemic IL-2 was not detectable in any animal. These results are in line with the PCR analysis above, suggesting that M3 cells are rapidly eliminated after injection.

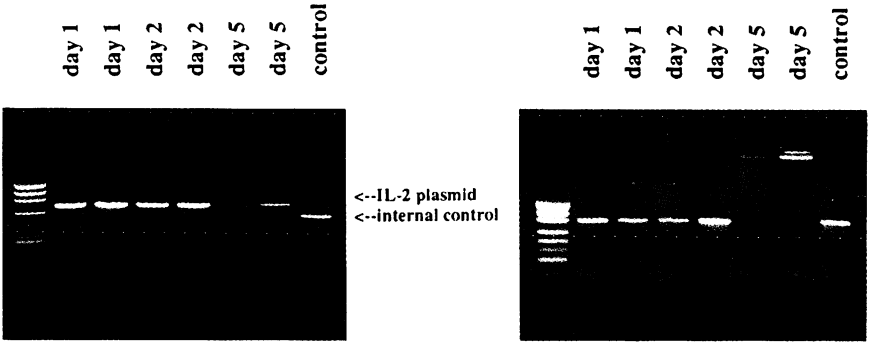

Fig. 2. PCR amplification of IL-2 plasmid from injection site: 3 x 10^5 IL-2 transfected cells were injected into DBA/2 mice. One, two and five days later, mice were sacrificed and immunization sites were excised. Tissues were incubated overnight in proteinase K buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) at 55° C. DNA was extracted twice with phenol /$CHCl_3$ followed by isopropanol precipitation. The PCR cocktail contained 1 µg DNA, 1 x PCR buffer (Boehringer Mannheim), 3 Units Taq-Polymerase (Boehringer Mannheim), 1 mM of each dNTP, and 25 pmol of specific primers. The conditions of standard PCR reaction were: 5 min denaturation at 95° C, followed by 40 cycles each 30 sec at 94°, 30 sec at 60° and 1 min at 72° C.

(Fig. 2a): As a control of IL-2 plasmid amplification (450 bp fragment), 1000 copies of a deleted version of the IL-2 plasmid were added to each PCR reaction, resulting in lower bands on the gel (280 bp fragment). Primers were 5' - G T C A A C A G C G C A C - C C A C T T C A A G C - 3' ; 5' - GCTTGTTGAGATGATGCTTTGACA -3'.

(Fig. 2b): Adenovirus amplification was proved by adding 1000 copies of adenovirus genome to the control reaction. Primers were 5' - GGTCCTGTG-TCTGAACCTGAG-3'; 5' -TTATGGCCTGGGGCGTTTACA-3' (317 bp fragment).

DISCUSSION

Our results clearly demonstrate that application of modified tumor cells expressing IL-2 at high levels leads to systemic and long-lasting protection against a challenge with highly tumorigenic cells. For modifying cancer cells with IL-2 genes, we have used a new gene delivery technique i.e. adenovirus-enhanced transferrinfection (AVET), which combines the receptor-mediated endocytosis uptake mechanism with the endosome disruption activity of adenovirus. This technique allows to test the efficiency of cytokine-production by the modified tumor cell over a wide range of expression of cytokines as demonstrated in our murine model. Most studies with cytokine-expressing tumor cells so far were performed with either stably transfected cell line or retrovirally transduced cells producing significantly lower levels (100-3.500 Units IL-2 /10^6 cells/24 h) than we routinely obtain. By using transferrinfection, transfected melanoma cells secrete up to 60.000 Units IL-2/10^6 cells/24 hrs. Our results show, that low levels of IL-2 (around 400 Units/ 10^6 cells/ 24 h) secreted from irradiated melanoma cells failed to protect completely. In contrast, the high production by the vaccine allowed to reject 1 x 10^5 parental melanoma cells in 100% of the immunized animals (see Table 1).

In contrast to stable integration of genes which is obtained by retrovirus-mediated transduction, AVET leads to long-term but transient expression of the delivered gene from episomally located DNA (Wagner et al., 1992). Besides persistence of gene expression, the survival of the cells after vaccination might be a limiting factor for application in humans since lethally

irradiated cells are applied. We therefore determined how long IL-2 has to be secreted and for how long the cells have to survive in vivo to induce an adequate immune response. Using PCR we detected amplifiable IL-2 or adenovirus DNA for 2 to animal 5 days after injection at the immunization site. These data indicate that neither transient cytokine expression nor irradiation is limiting for the induction of the immune system. Another important finding is that no IL-2 DNA nor adenovirus DNA is detectable in draining lymph nodes or other tissues. These data also show that modified tumor cells do not migrate and exclude the possibility of direct gene transfer into the germ line by the applied transfection and immunization procedure.

AVET for the generation of "tumor vaccines" is not only restricted to melanoma cancer or to IL-2. Other specific targets like modified colon, liver, or renal tumor cells should also lead to enhanced immunogenicity against developing metastases when applied under the appropriate conditions. The general concept for the stimulation of the host immune system through elevated levels of cytokines produced at the immunization site seems to be more dependent on the ctyokine level than on the time period of exposure. Further experiments need to be done to investigate the host immune response to the induced cytokine expression and the resulting characterization of cells invading the tumor and the resulting change in the specific cytokine response at the immunization site will lead to further understanding the role of the induced ctyokine and the importance of high expression levels.

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