Abstract. Oncolytic viruses have emerged as a novel class of potent anticancer agents offering an improvement on chemotherapy and radiotherapy in terms of tumor targeting and reduction of side-effects. Among these agents, autonomous parvoviruses have attracted the attention of researchers for their ability to preferentially replicate in and kill transformed cells, and to suppress tumors in the absence of adverse reactions in various animal models. We have previously shown that lethally irradiated autologous tumor cells can support parvovirus H-1PV production and serve as carriers to deliver progeny H-1PV into the vicinity of lung metastases in a rat tumor model, resulting in H-1PV infection of and multiplication in metastatic cells. It is known that irradiated autologous (neoplastic) cells can also act as a therapeutic vaccine against the original tumor. Yet the ability of these cells to suppress metastases in the above model was found to be much increased as a result of their H-1PV infection. This prompted us to determine whether H-1PV boosted the tumor-suppressing capacity of the autologous vaccine by increasing its immunogenic potential and/or by making it a factory of oncolytic viruses able to reach and destroy the metastases. Both effects could be dissociated in the presence of neutralising antibodies which either prevent the progeny viruses from spreading to metastatic cells, or deplete the CD8 effector cells from the immune system. This strategy revealed that the H-1PV infection of tumor cells enhanced their ability to trigger an immune response for which uninfected tumor cells could be the targets, thereby amplifying and taking over from the direct viral oncolytic activity. This dual oncolytic/vaccinal effect of H-1PV holds out promises of clinical applications to cancer therapy.

Introduction

Metastases represent the most dangerous complication associated with cancer progression. Very often the lung is one of the first organs to be seeded by cells migrating from different primary malignancies (1). Prevention or treatment of this deadly spread has been the aim of many therapeutic protocols using different strategies (2-4). Several members of the genus Parvovirus (H-1PV, MVM, LuIII) are presently under consideration for cancer gene therapy applications (5) due to their ability to preferentially propagate in (oncotropism) and kill (oncolysis) neoplastically transformed cells (6,7), and their capacity for the asymptomatic infection of humans. MVM and H-1PV have been shown to exert oncosuppressive activities in vivo, i.e. they inhibit the formation of spontaneous, chemically or virally induced tumors in laboratory animals (8). In particular, we have recently shown that H-1PV is able to suppress metastases in a rat tumor model (9). Assuming that the treatment with oncolytic parvoviruses would be more efficient if the virus was delivered locally to metastases instead of systemically, we devised a strategy using lethally irradiated infected autologous tumor cells as carriers. After intravenous (i.v.) injection in rats bearing lung metastases, these cells were indeed found to home into the lungs, and to produce and deliver H-1PV progeny virions that could spread to neighbouring metastatic cells, resulting in a more efficient suppression of metastases as compared to treatments with free H-1PV or mock-infected carrier cells (9).

It is known that the anticancer activity of certain oncolytic viruses, notably the Newcastle disease virus (NDV) (10), is not only mediated by their direct killing effect on cancer cells, but also involves an adjuvant effect of the virus on the immune system (11,12). Besides ‘exposing’ tumor-associated antigens, oncolytic viruses can provide danger signals and/or modulate the production of immunoregulating molecules, resulting in the activation of the immune system and the breakage of tolerance towards the tumor. The question arose
whether in the above metastatic rat model, H-1PV exerted its oncosuppressive effect solely through the direct lysis of target tumor cells, or also by enhancing the ability of autologous tumor cells serving as carriers to act as a therapeutic vaccine. In order to distinguish between these two possibilities, it was determined whether the prevention of H-1PV spreading from the carrier cells to target metastases impaired the protection provided by the parvovirus against the formation of metastatic nodules. The infection of animals or humans with parvoviruses leads to seroconversion and the production of neutralising antibodies (13). Rats were therefore preimmunised against H-1PV so as to contain antiviral neutralising antibodies at the time of the induction of metastases and treatment with autologous carrier cells. These antibodies proved able to abolish any detectable infection of metastatic cells by viruses released from the carrier cells. Yet, the parvoviruses inoculated to the carrier cells were still able to exert an oncosuppressive activity under these conditions, for which uninfected metastases were the targets. This indirect protective effect proved to be immune-mediated, showing that the H-1PV infection of lethally irradiated autologous tumor cells enhances their vaccinal potential. The oncosuppressive capacity of carrier cell-delivered H-1PV was however, higher in the absence of neutralising antibodies, when the infection of target metastases could take place. Altogether these data give striking evidence to suggest that H-1PV exerts a direct oncolytic effect, which is then amplified or relayed by a virus-boosted antitumoral immune response.

Materials and methods

**Cell culture.** The Morris hepatoma 3924A cell line (MH) was a gift of Dr Uwe Haberkorn (Department of Oncological Diagnostics and Therapy, German Cancer Research Center, Heidelberg, Germany). The AS-IB1 cell line (ASML) was kindly provided by Dr Margot Zöller (Department of Tumor Progression and Immune Defense, German Cancer Research Center, Heidelberg, Germany) (14). MH and ASML cells were maintained in RPMI-1640 medium containing 20% fetal calf serum (FCS). The SV40-transformed newborn human kidney cells (NBK) were cultured in MEM supplemented with 5% FCS. The culture media were supplemented with penicillin (100 μg/ml) and streptomycin (100 U/ml). All the cells were kept at 37˚C in a 5% CO₂ atmosphere.

**Titration of virus-neutralizing antibodies (αH-IPVNAb).** αH-1PVNAb titers were determined as previously described (9). Shortly, serial dilutions of the sera from experimental animals were made in MEM and mixed with an equal volume of H-1PV virus suspension [corresponding to 2x10⁴ replication units (RU)/well]. After incubation for 30 min at 37˚C, the mixture was inoculated to NBK cells plated in 96-well dishes (2x10³ cells/well). The cell survival rates were assessed 72 h after using the MTT assay. Virus neutralizing titers were expressed as serum dilutions that protected cell cultures from virus-induced killing.

**Irradiation.** The γ-irradiation of MH and ASML cell cultures was performed using a Cs-137 Gammacell 1000 γ-irradiator at a dose of 30 Gy.

**Virus production and titration.** The H-1PV virus was produced in NBK cells, purified through iodixanol gradient centrifugation and tested for its infectivity through the formation of replication centers in NBK indicator cell monolayers, using a previously described hybridization assay (7). Viral titers are expressed as RU. The titration of the viruses from the lung tissues was performed in the same way by applying dilutions of clarified lung homogenates on NBK monolayers.

**Virus expression.** For the analysis of viral transcription in the organs of virus or carrier cell-treated animals, total RNA was extracted from the samples of collected tissues (kept at -80˚C) using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA and analysed using the RT-PCR protocol, described previously (15). The pair of primers used revealed H-1PV DNA or R1 transcripts in the form of 512- and 415-bp PCR fragments, depending on the excision of the small intron: 5'-TCAATGGCCTACCATCTCGT-3' (forward) and 5'-TCGATGGCTTCTGGTGGTCT-3' (reverse). Sample matching was verified by quantifying the β-actin transcripts with primers generating a 489-bp PCR fragment: 5'-ATGGATTGACCTCTCAACAC-3' (forward) and 5'-ACGTACACCTTCATGTATGC-3' (reverse). The sensitivity of the method was established to be about 10 copies of viral transcripts or ssDNA genomes per μg RNA.

**Animal treatment and metastatic model.** Eight-week-old ACI rats weighing 200-220 g (Harlan, Gannat, France) were housed under conventional conditions (a temperature of 20-22˚C, and a dark-light rhythm of 12 h), receiving food and water ad libidum. All experiments were performed in compliance with European and local guidelines. Prior to all the procedures, the animals were anesthetized with isoflurane (Forene®, Abott Laboratories, Rungis, France).

For the induction of lung metastases, MH cells harvested by trypsinization, were washed with PBS and injected into the femoral vein of the rats (1x10⁵ cells per animal) in a volume of 300 μl PBS.

The carrier cells (MH or ASML) were prepared as previously described (9) except for the irradiation dose that was increased to 30 Gy in order to ensure cell killing and optimize the vaccinating effects. Briefly, the cultures were inoculated with H-1PV at a multiplicity of infection (MOI) of 3 RU per cell, incubated for 24 h, harvested and irradiated in suspension. These so-called carrier cells were then injected intravenously into the rats (5x10⁶ cells in 300 μl PBS per animal).

The induction of αH-1PVNAb was performed through the intraperitoneal inoculation of H-1PV (1x10⁶ RU per rat) 3 weeks prior to metastases induction.

CD8 lymphocyte depletion was achieved by two successive injections of CD8-specific monoclonal antibody (OX-8; ECACC, Salisbury, UK) first intravenously at the time of vaccination with the carrier cells, then intraperitoneally 7 days later (1 mg per animal in total). The purification of the OX-8 antibody from the hybridoma supernatant and the depletion experiments were carried out according to previously described protocols (16,17).
For assessing the numbers of metastatic nodules, rats were sacrificed under anaesthesia through exsanguination. A blood sample (500 μl) was kept and centrifuged after clotting to collect the serum that was stored at -20°C for further analysis. After opening of the thoracic cavity, the trachea was canulated with a syringe, and the lungs were insufflated with 5-6 ml 5% Indian ink solution in 0.9% NaCl. The white metastatic nodules (size 1-2 mm) were visualized on the black back-ground of the lung tissue, after submerging the lungs in Fekette’s solution (58% ethanol, 3% formaldehyde, 0.04% glacial acetic acid). The nodules on the surface of the organ were counted using a magnifying lens. Lung draining lymph nodes (dLN) were extirpated and passed through a mesh to prepare cell suspensions in RPMI.

Quantification of blood lymphocyte subset. Leukocytes were isolated from fresh heparinised rat peripheral blood and prepared for flow cytometry analysis. Indirect and direct staining were performed by 30-min incubation with the monoclonal antibodies against CD3, CD4 and the OX-8 (αCD8) antibody, which recognized T helper/suppressor subsets, B cells and cytotoxic lymphocytes, respectively. After washing, incubation with secondary antibodies was carried out for 30 min, using FITC-conjugated rabbit anti-mouse IgG and IgM antibodies. All reagents were purchased from BD Biosciences, Europe. The cells (10⁶) were analysed on a FACScan flow cytometer (Becton-Dickinson, USA) using CellQuest software.

Statistical analyses. Mean and standard deviations were calculated for cell survival, and metastases incidence data. The statistical significance of the differences in the incidence of metastases displayed by the individual groups of treated animals was assessed using a one-way analysis of variance, followed by the parametric Student’s unpaired t-test. The correlation between the numbers of lung metastases and the cellularity of dLN was studied using the parametric Pearson’s test. A difference between individual values was considered significant when the P-value was below 0.05. The Instat 2.00® Macintosh software (GraphPad Software, San Diego, CA, USA) was used for these analyses.

Results

Tumor cell carriers of H-1PV can stimulate the suppression of autologous neoplastic cells without causing their infection. H-1PV delivered as a naked virus or released from infected carrier cells has previously been shown to exert a direct oncolytic effect on rat hepatoma (MH) cell cultures, and to suppress pre-established metastases developing from these cells in the lungs of immunocompetent ACI rats (9). These observations raise the question, as to what extent metastatic cells undergoing suppression in vivo are directly killed by H-1PV and/or are targets for an immune response that is primed by the virus but acts on uninfected tumor cells. In order to test the ability of autologous tumor cells serving as carriers for H-1PV, to function as a therapeutic vaccine, we designed a protocol in which the spreading of viruses produced by these cells to the tumor cells residing in the lungs could be interrupted. As depicted in Fig. 1, the animals were either primed or not with H-1PV three weeks before the inoculation of the metastatic cells (day 21). After the establishment of metastases, H-1PV-infected and irradiated carrier cells were injected (day 27), and three weeks were allowed for the induction of any oncolytic and vaccinal effect prior to the measurement of the incidence of lung metastases (day 50).

As shown on Fig. 2A, H-1PV-neutralising antibodies (αH-1PVNAb) were present in high titers in the serum of primed rats at the time of the implantation of the metastatic MH cells. There was no evidence of H-1PV persistence in these animals as is apparent from the lack of detectable (RT)PCR signal for viral DNA and transcripts in any of the tissues tested at day 21 (<10 copies per μg RNA) (Fig. 2B, lower panel). Virus clearance was further supported by the fact that the incidence of MH cell-derived lung metastases was similar in primed vs unprimed animals in the absence of further treatment (data not shown). This indicated that at the time of the MH cell injection, primed rats did not harbour anymore infectious H-1PV viruses liable to interfere with the growth of MH metastases. Primed and naïve animals subjected to the MH cell injection (day 21) and the onset of lung metastases growth, were treated at day 27 with H-1PV-infected and irradiated carrier cells, and analysed for the infection of metastatic tissues with viruses produced and released by the carriers. Two types of cells were used as carriers: Autologous MH cells and allogenic ASML cells. Both carriers were previously shown to home into the lungs of rats (14) and to produce similar titers upon irradiation (9). The multiplication of H-1PV in the metastatic lesions was measured by titrating the virus from the lung homogenates, using a replication-centre hybridisation assay on NBK indicator cells. As illustrated in Fig. 2C (αH1PVNAb), infectious H-1PV
was readily detected in the lungs of naïve tumor-bearing rats following their treatment (day 27) with free virus or either virus-carrying cells. This virus load has previously been shown to depend on the multiplication of input virions and the presence of MH metastases in lung tissues (9). In contrast, the administration of H-1PV or infected carrier cells (day 27) to the primed animals did not cause the infectious virus to accumulate in the lungs (Fig. 2C, +αH1PVNAb).

αH-1PVNAb thus proved able to prevent the virus from infecting and multiplying in the target metastatic cells, irrespective of whether H-1PV was delivered systemically (free H-1PV) or locally (carrier cells). In agreement with these data, the primed rats did not harbour any detectable viral nucleic acids in the lungs following their infection with free H-1PV at day 27, as assayed by (RT)PCR, while the primed animals treated with carrier cells displayed only a weak PCR signal ascribable to the lung-seeding carriers themselves (data not shown).

The question was then addressed whether H-1PV could still suppress the formation of metastases to the same extent in the primed rats i.e. under conditions minimising the direct infection of the target tumor cells and ensuing the oncolytic effect of the virus. As illustrated in Fig. 3 (panels A and B, group 1), free H-1PV had no protective effect in continuing with its seroneutralisation and resultant inability to infect any cellular player in the oncosuppressive process. In contrast, the infection of MH carrier cells enhanced their capacity for inhibiting the formation of metastases Fig. 3 (panels A and B, groups 2 & 3). Therefore, H-1PV was able to stimulate the suppression of presumably uninfected tumor cells when the virus was produced by irradiated cancer cells serving as carriers. Since lethally irradiated neoplastic cells can work as
a therapeutic vaccine against an autologous tumor (12), these results raised the possibility that the H-1PV infection of such a vaccine could enhance its anticancer immunogenic potential. This prompted us to further test whether the H-1PV-dependent oncosuppressive activity of autologous carrier cells in the presence of αH1PVNAb was indeed immune-mediated.

Therapeutic vaccination contributes to the oncosuppressive activity of H-1PV. Should the H-1PV infection of carrier cells stimulate their vaccinal effect, the antimetastatic activity of the virus would be expected to depend on the use of carrier cells that are autologous to the target tumor cells. As illustrated in Fig. 3A (group 4), this prediction was verified by showing that H-1PV failed to endow the allogenic ASML carrier cells with a capacity for suppressing MH metastases. Similar to MH cells, ASML cells are permissive for H-1PV infection and competent for transmitting H-1PV to MH metastases in the absence of neutralising antibodies (Fig. 2C). Therefore, the inability of infected ASML carrier cells to achieve any significant protection against MH tumors in primed animals, confirmed that the neutralising antibodies were efficient at preventing direct virus-induced oncolysis. In primed animals, the potential antih-MH metastasis activity of irradiated MH cells as a result of their H-1PV infection, was most likely due to an adjuvant effect of the virus on the autologous vaccine.

Direct evidence of the involvement of the immune system in the H-1PV enhanced capacity of irradiated MH carrier cells for suppressing MH metastases was sought by treating tumor-bearing animals with αCD8 antibodies at the time of and after the injection of the carrier cells. αCD8 antibodies introduced either intravenously or intraperitoneally have been shown to successfully shut off the effector arm of the adaptive immune system by depleting CD8+ cytotoxic lymphocytes (16,17). Indeed, a drastic CD8 cell depletion was achieved in the present system by treating animals with CD8-specific monoclonal antibodies, as assessed by FACS of CD3- and CD8-positive cells (Fig 4A, compare the - and +αCD8 panels). In order to evaluate the relative contribution of direct viral oncolysis and virus-enhanced vaccination to the overall anticancer effect of systemically applied H-1PV-carrying autologous cells, four groups, of 6 animals each, were treated as indicated at the bottom of Fig. 4B. According to the experimental scheme depicted in Fig. 1, all the rats were inoculated with metastasis-inducing MH cells at day 21, and with H-1PV-infected and irradiated MH cells, at day 27. Group 1 was further depleted of CD8 cells to reveal the direct oncolytic activity of H-1PV delivered through the MH
carriers in the absence of the vaccination effect. Conversely, group 4 was primed to produce αH1PVNAb and thereby display the vaccinal function of the H-1PV-carrying cells in the absence of viral infection and lysis of target metastatic cells. Rats from group 2, serving as the negative controls, were both lympho-depleted and primed to produce αH1PVNAb with the aim to switch off both therapeutic effects. Group 3, on the contrary, did not receive any of the latter treatments in order to take advantage of both the oncolytic and vaccination components of the H-1PV oncosuppressive activity. As shown in Fig. 4B (compare groups 2 and 3), the treatment of tumor-bearing rats with autologous carrier cells under conditions allowing H-1PV to exert both activities (αH1PVNAb, -αCD8) led to a striking reduction in the incidence of metastases. Primed animals, in which neutralising antibodies prevented H-1PV from having a direct oncolytic action, showed a much lesser, yet significant protection against metastases formation after treatment with infected carriers (group 4). As stated in the previous section, this residual protection was dependent to a large extent on the infection of carrier cells with H-1PV, and was assumed to be immunemediated. The present data confirmed this assumption by showing that abrogation of the host cytotoxic T-cell response (group 1) reduced the oncosuppressive activity of infected carrier cells (compare with group 3) to a residual level ascribable to the direct oncolytic effect of the released virus (compare with group 2). In this experimental system, in which permissive autologous tumor cells were used as carriers, H-1PV therefore had both direct (oncolytic) and indirect (adjuvant) effects that were of similar levels and acted in a cumulative fashion to achieve an overall strong inhibition of metastatic growth.

In order to further substantiate the occurrence of an immunological component in the antimitastatic protection provided by H-1PV-infected autologous carrier cells, dLN were collected from treated rats that had not been exposed to αCD8 monoclonal antibodies. The abundance of lymphocytes (cellularity) was then determined in dLN as an index of local immune activation in these animals. As shown in Fig. 4B, an inverse correlation was observed between the dLN cellularity and metastases rate in individual carrier cell-treated rats. The data fitted a negative slope regression line, which was not the case with the untreated animals, and was indicative of a participation of the immune system in the H-1PV-dependent, carrier cell-mediated suppression of metastases.

Discussion

The results described above give strong evidence to suggest that the H-1PV infection of tumor cells (including carrier cells in the present system) contributes to reduce the tumor load not only by causing the direct lysis of neoplastic cells but also by stimulating their recognition as a therapeutic vaccine by the immune system. The latter immunomodulating capacity of H-1PV is supported by recent reports showing that the infection of human melanoma cells with this virus results in the activation of cocultured dendritic cells and the ensuing cross-priming of a cytolytic T-cell response (18). Since H-1PV-infected melanoma cells were more efficient than the corresponding uninfected cell lysates in achieving this immune activation, it was hypothesised that the virus can induce tumor cells to release immunostimulating factors and/or kill them in a way that promotes the uptake of tumor-associated antigens (19).

The use in the present study of irradiated autologous cells as H-1PV carriers, proved suitable to achieve both parvoviral effects (namely direct oncolysis and vaccination) through a single treatment. For the sake of increasing antitumor efficiency, the sequential application of H-1PV at first, and of H-1PV-infected autologous carrier cells after that, is worth considering in a clinical setting. One advantage of rodent autonomous paroviruses in comparison with most of the viruses used in virus and gene therapy protocols, lies in the absence of widespread, if any, antiviral immunity in the human population (20). This leaves a broader therapeutic window for applying free H-1PV within a time interval of 7-10 days, i.e. before the onset of αH1PVNAb production, and can be expected to provide a first level of anticancer protection in humans, by analogy with the effects seen in animal models (21,22). Since further injections will be inefficient due to virus neutralisation, the therapeutic treatment could then be continued by inoculating H-1PV-infected irradiated autologous tumor cells, in order to take advantage of the vaccinal arm of the viral oncosuppressive activity. Such a stepwise protocol would allow the optimal exploitation of the lytic and adjuvant effects of H-1PV. It should be stated that the second step in this regimen can be expected to be not only unhampered but possibly promoted by the immune responses triggered by the first step (i.e. injection of the free virus). Besides a non-specific immune stimulation resulting from virus infection, the oncolytic activity of inoculated virions should indeed prime the host to the booster treatment with irradiated autologous H-1PV-carrying cells. In addition to the direct destructive effect in the tumor, the first administration of free viruses could in this way lead to stronger cellular immune reaction to the viral and tumor-associated antigens displayed by subsequently inoculated autologous H-1PV carriers, thereby improving their vaccination potential through the antiviral recall immune response.

It is presently unclear how the anti-tumor vaccinating capacity of H-1PV compares with the one displayed by other viruses, in particular NDV which is endowed with striking immuno-stimulating features (10-12). It is worth noting in this respect that distinct strategies are available to modulate the interplay between H-1PV and the immune system. One approach consists of supplementing the virus with foreign transgenes that encode immunostimulating molecules. Parvoviral vectors were produced in this way to transduce various chemo- and cytokines (5,7). Some of the recombinants proved to be more efficient than the wild-type parental virus in their ability to suppress specific tumors, which correlated with the infiltration of neoplastic tissues with immune cells (21,22). Another strategy, under current investigation in our laboratory, consists of modifying parvoviruses so as to increase their recognition as danger signals by the innate immune system, with the object of activating antigen-presenting cells in the vicinity of tumors that sustain virus production.

Altogether these data lead to the conclusion that the H-1PV-dependent oncosuppression cannot be merely assigned to direct virus-induced oncolysis and therefore involves, in
addition to the latter process, an immune component in which the parvovirus also has an active part. These oncolytic and adjuvant features of the virus can both be improved through appropriate genetic modifications. Therefore these agents, in particular H-1PV, have, in our opinion, a great potential for enriching the armament of clinical anticancer protocols and achieving a direct decrease in the tumor burden from which a tumor-specific therapeutic vaccination can take over.

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