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Commentary

Antiproliferative prostaglandins and the MRP/GS-X pump role in cancer immunosuppression and insight into new strategies in cancer gene therapy

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Abstract

A dramatic complication in late-stage cancer patients is host immunosuppression. Cyclopentenone prostaglandins (CP-PGs) overproduced in cancer may impair the function of the immune system. These agents, if produced at high concentrations, are powerful cytostatic and cytotoxic compounds that may arrest cell proliferation and immune response in cancer. Lymphoid tissues of tumor-bearing animals accumulate large amounts of CP-PGs, whereas the tumor tissue does not. This may be because cancer cells are able to overexpress multidrug resistance-associated protein (Mg^{2+}-dependent vanadate-sensitive G*S*-conjugate export ATPase, MRP/GS-X pump), which extrudes CP-PGs to the extracellular space as glutathione *S*-conjugates. In contrast, MRP/GS-X pump activity is disproportionately low in lymphocytes. This led us to propose the transfection of lymphocytes with multidrug resistance-associated protein genes (*MRP*) for further autologous transfusion or direct *in vivo* delivery to lymphocytes by using adenovirus-retrovirus chimeras in order to restore immune system function in cancer, at least partially. We are currently evaluating MRP-transfected lymphocyte (MTL) therapy, using Walker 256 tumor-bearing rats as a model.

Keywords: Multidrug resistance; MRP/GS-X pump; Antiproliferative prostaglandins; Glutathione metabolism; Cancer chemotherapeutics; Cancer immunosuppression

1. Introduction

Cancer-associated immunosuppression is a common complication and reason for the poor prognosis of late-stage cancer patients. The tumor itself plays a role in the development of immunosuppression through the production of substances that impair the function of the immune system. Among known immunoblocking substances, PGs may be of importance in dictating the degree of immunosuppression and, thus, the rate of tumor growth.

The plasma of tumor-bearing humans [1] and other mammalian models [2] exhibits high levels of PGs, especially those of the E-type (mainly PGE_2). This is because tumor cells [2,3] as well as specialized antigen-presenting cells [4], stimulated by the manifestation of an initiating tumor, produce PGE_2 and PGD_2 [5,6], whose presence is associated with the impairment of immune function, thus leading to immunosuppression and cancer cachexia [4,7]. *In vitro* studies also showed that PGE_2 released by HEp-2 human larynx carcinoma cells inhibited human polymorphonuclear leukocyte migration, which was reversed by treating HEp-2 cells with the non-steroidal anti-inflammatory indomethacin, a PG synthesis inhibitor [3]. PGE_2 reduces lymphocyte proliferation and promotes unresponsiveness to antigen challenge in normal B lymphocytes [8]. PGD_2 produced by tumor cells has also been reported to be antiproliferative for different cell types [7].

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Abbreviations: ABC, ATP-binding cassette transporter ATPase; CP-PG, cyclopentenone prostaglandin; GSH, glutathione; GSSG, glutathione disulfide; G*S*-conjugate, glutathione *S*-conjugate; MRP/GS-X pump, Mg^{2+}-dependent vanadate-sensitive G*S*-conjugate export ATPase; hsp, heat shock protein; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; MTL therapy, MRP/GS-X pump-transfected lymphocyte therapy; NF-κB, nuclear factor kappa B; PG, prostaglandin; and PPAR-γ, peroxisome proliferator-activated receptor-gamma.

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In this commentary, the possibility of reversal of PG-based immunosuppression in cancer is presented as an alternative strategy in cancer therapeutic practice.

2. CP-PGs

Although PGE_2 and PGD_2 may be antiproliferative and immunosuppressive *per se* in cancer, they are enzymatically transformed into, respectively, PGA_2 and J_2-series PGs (PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2) by exposure to plasma, serum, or solutions containing albumin [9]. PGs of the A- and J-types, characterized by the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring, are therefore collectively named CP-PGs and appear to be the actual effectors of PG-mediated immunosuppression in cancer [10] (see Fig. 1 for the CP-PG metabolic pathway and chemical structures). Indeed, almost the entire antiproliferative effect previously observed for PGE_2 and PGD_2 may be ascribed to their CP-PG derivatives. CP-PGs, which were thought for many years to be artifacts of tissue sample preparations, are actually formed *in vivo* and may be detected in body fluids and tissues of both humans [11] and experimental animals [12]. Additionally, CP-PGs of the J-series are physiological ligands for the nuclear transcription factor PPAR-γ [13,14]. PPAR-γ is an important transcription factor related to cell proliferation and differentiation, and the serendipitous discovery of its metabolic activation by CP-PGs has shed light on its pivotal role in glucose and lipid homeostasis as well as its potential relevance in metabolic disorders such as diabetes and obesity. The promising value of CP-PGs as cancer cell differentiation agents through PPAR-γ activation, however, remains to be established.

CP-PGs inhibit cell proliferation in a variety of experimental tumor models, *in vitro* as well as *in vivo* [15]. They are actively and selectively transported into cells by a cell membrane carrier and then transferred to the nuclei where they bind to nuclear proteins and specifically act at the G_1/S interface of the cell cycle [10,15]. Depending upon the concentrations employed, CP-PGs may be extremely cytotoxic by blocking protein synthesis and causing damage to the actin filaments of the cytoskeleton [16], while they inhibit the expression of DNA polymerases β and γ [17]. The antiproliferative effect of CP-PGs depends upon their uptake by cells, a phenomenon which may be so effective that it is difficult to detect such substances *in vivo* or a few minutes following addition to the culture medium [18]. Moreover, inhibition of cell proliferation by CP-PGs is dependent upon the presence of at least an α,β-unsaturated carbonyl group (see Fig. 1) and is associated with the induction of a 70-kDa heat shock protein (hsp70), through the cycloheximide-sensitive activation of the heat shock transcription factor (HSF) [10,15]. In addition, CP-PGs inhibit the activation of the nuclear transcription factor κB (NF-κB) by direct inhibition of IκB kinases through the reaction of CP-PGs with cysteine residues of such kinases, which are necessary for the activation and DNA binding activity of NF-κB [19]; this effect is also associated with HSF activation [20,21]. Since NF-κB activation triggers the transcription of a myriad of genes involved in the immune response, cell proliferation, and differentiation, the CP-PG-mediated block of NF-κB activation may play a role in cell growth inhibition and immunosuppression.

The antiproliferative effect of CP-PGs is such that they were proposed as an alternative treatment in cancer chemotherapy. The lack of specificity and the inhibitory effect on immune cells, however, precluded this usage.

3. Interconversion of PGs and cancer immunosuppression

The observation that the plasma of cancer-bearing subjects may carry large amounts of PGE_2 and PGD_2 and that these PGs are easily transformed into CP-PGs led to the proposition that the interconversion of PGs might be responsible, at least partially, for the dramatic immunosuppression of late-stage cancer patients (see Fig. 2 for a summarized explanation). Such a possibility was tested in this laboratory by using Walker 256 tumor-bearing rats as a model [12]. This tumor is a carcinosarcoma which characteristically causes immunosuppression and cachexia [22]. Walker 256 tumor-bearing rats show high plasma levels of PGE_2 [2]. Administration of sub-antiinflammatory doses of aspirin-like drugs (PG synthesis inhibitors) to these rats dramatically reduced tumor growth, improving food intake, body weight gain, and insulin secretion [23,24]. Also, immune tissues from these animals accumulated large amounts of the CP-PGs (PGAs) [12], which is not the case, however, for the tumor tissue itself, where CP-PGs are not detected. This raises a question: if the interconversion of PGs into CP-PGs occurs in the plasma of tumor-bearing subjects, how does one account for the accumulation of CP-PGs only in immune tissues of such subjects? This is especially intriguing considering that all eukaryotic cells tested thus far take up CP-PGs, being sensitive to their antiproliferative effects. The characterization of a membrane ATPase activity (GS-X pump), responsible for the extrusion of hydrophobic electrophiles from cells as *S*-conjugates [25], provided a clue for the answer to this question.

4. Physiology of the transport through the MRP/GS-X pump and multidrug resistance in cancer

Plant and animal cells eliminate a broad range of hydrophobic toxins from the cytosol to the extracellular space after their conjugation with GSH (for a review, see Ref. 25). This transport is mediated by a novel class of organic anion transporters belonging to the family of ATP-binding cassette (ABC) carriers, the GS-X pumps [26]. These primary

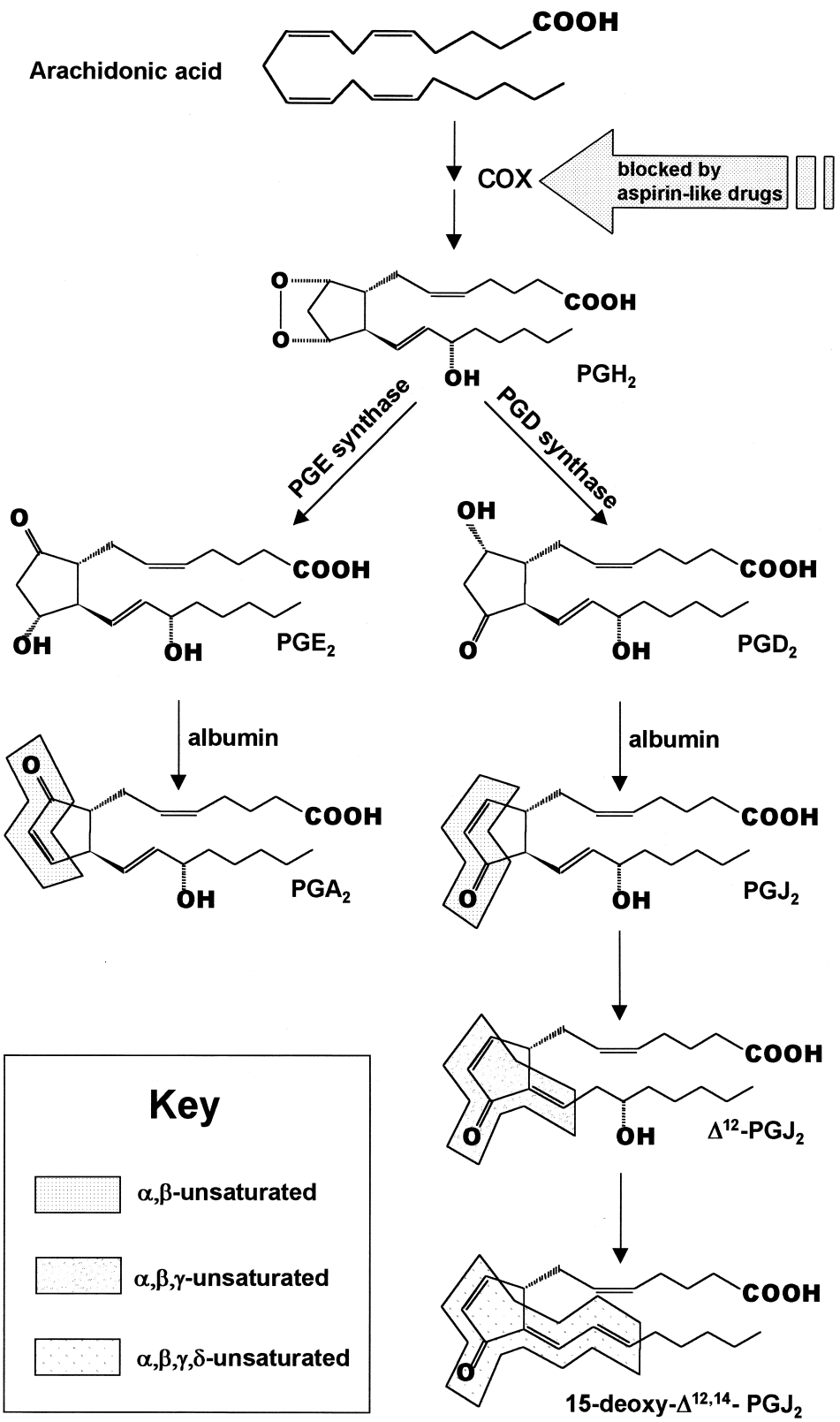

Fig. 1. The CP-PG metabolic pathway and chemical structures. In mammalian cells, arachidonic acid is the main precursor of PGs, although other 20-carbon essential fatty acids (eicosatrienoic and eicosapentenoic acid) may also generate PGs through the two-step aspirin-like drug-sensitive cyclooxygenase (COX) catalyzed reaction. After arachidonic acid delivery from membrane phospholipids (the major store) and prompt conversion to PGH_2 via COX, parental E- and D-type PGs are synthesized through PGE and/or PGD synthases, depending on the cell type. In the presence of albumin, these parental PGs may be transformed into the CP-PGs PGA_2 and J_2-series PGs, respectively. The α,β-unsaturated electrophilic centers, obligatory for CP-PG biological effects, are also shown.

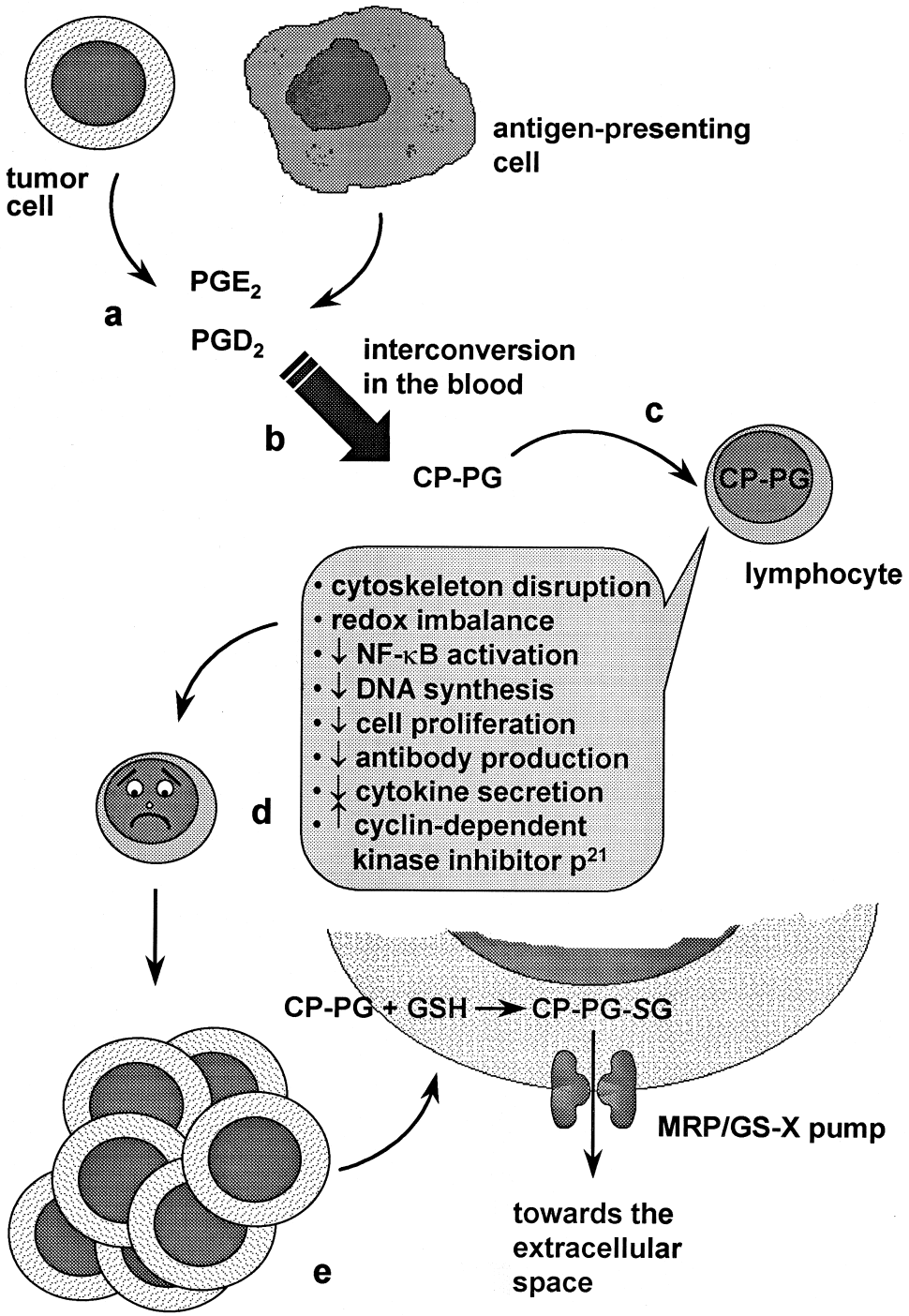

Fig. 2. Impact of CP-PG production on host immune system function in cancer subjects. (a) Tumor cells as well as antigen-presenting cells stimulated by the presence of the initiating tumor produce large amounts of E- and D-type PGs, which may be (b) converted into CP-PGs in the host blood. (c) CP-PGs are promptly taken up by lymphocytes where they cause dramatic changes in lymphocyte cell physiology leading to (d) immunosuppression. (e) In the absence of an effective immune response, tumor cells can preferentially proliferate in the late stages of cancer. Contrary to that observed in lymphocytes, tumor cells strongly express the MRP/GS-X pump, which eliminates CP-PGs towards the extracellular space as GS-conjugates, remaining free from the cytostatic/cytotoxic effects of CP-PG.

active transporters are Mg^{2+}-dependent vanadate-sensitive export ATPases that extrude GS-conjugates and exhibit high affinity towards GS-conjugates carrying a long aliphatic carbon chain [26]. The term GS-X pump was originally coined by Ishikawa [25] to describe the ability of such carriers to extrude hydrophobic xenobiotic substances, albeit GS-X pumps are the physiological transporters of naturally occurring GS-conjugates, including GSSG and leukotriene C_4 [25–27]. GS-X pump activity was localized in canalicular and basolateral rat hepatocyte plasma mem-

branes, heart sarcolemma vesicles, human erythrocytes, and tumor cells [26,28], while defective GS-X pump activity accounted for conjugated hyperbilirubinemia in TR^- mutant Wistar rats [29], a phenotype similar to that found in the human hyperbilirubinemia II/Dubin-Johnson syndrome.

Inasmuch as GS-X pumps extrude G*S*-conjugates of anticancer electrophiles, such as cisplatin, and other unmodified antitumor drugs such as vinblastine and methotrexate, they prompt a threat to cancer therapeutic approaches by causing resistance because the drugs do not accumulate in tumor cells as expected. Also, GS-X pump activity is overexpressed in cisplatin-resistant human leukemia, thus causing acquired resistance to a number other antitumor electrophiles (for a review, see Ref. 26).

Tumor cells may overcome the challenge of anticancer drugs and biologically active hydrophobic substances of an endogenous nature by exporting them via different unidirectional efflux systems. Several ABC membrane transporters have been described in this context, and these export proteins may account for the elimination of antiproliferative substances. The ABC transporter family comprises a variety of carriers involved in the expression of the multidrug resistance (MDR) phenotype, including the small subfamily of 170-kDa plasma membrane glycoproteins (P-glycoproteins or P-170) encoded by the *MDR* genes [30]. Apart from these gene products, another member of the ABC transporter family has been characterized recently as a non-P-glycoprotein carrier associated with the MDR phenomenon [31]. Interestingly, this novel MDR-associated protein (MRP) resembled, in many aspects, the GS-X pumps. Then it became clear that the 190-kDa *MRP1* gene product was a GS-X pump [32]. As featured by GS-X pumps, MRP transports a variety of G*S*-conjugates, including leukotriene C_4, in an ATP-dependent manner [33]. Many drug-resistant cancer cell lines exhibit a notable MRP-dependent efflux of G*S*-conjugates [34,35], while transfection with and overexpression of the *MRP1* gene alone is sufficient to cause a non-P-glycoprotein-mediated MDR [28,32]. In contrast, co-transfection of MDR1 and MRP1 antisense RNAs has been shown to abolish drug resistance in multidrug-resistant human lung cancer cells that overexpress both *MDR1* and *MRP1* genes [36]. Five additional *MRP* homologous genes (*MRP2–6*) including new hepatic canalicular multispecific organic anion transporter (cMOAT) homologues have been cloned recently in different cell lines in which the expression of such genes appears to correlate with drug resistance. Hence, at the moment, at least six *MRP* homologues are known: *MRP1* (or MRP), *MRP2* (also called cMOAT), *MRP3* (also called MOAT-D), *MRP4* (also called MOAT-B), *MRP5* (also called MOAT-C), and *MRP6* [37–39]. Also, recent evidence suggests that the MRP/GS-X pumps may co-transport unmodified anticancer drugs (e.g. vinca-alkaloids) together with GSH [39].

5. Resistance to CP-PG antiproliferative effects and the role of the MRP/GS-X pump in cancer immunosuppression

The α,β-unsaturated carbonyl group of CP-PGs (see Fig. 1) is electrophilic and susceptible to covalent binding to the thiol group of the cysteine of GSH via a glutathione *S*-transferase-catalyzed reaction, forming inactive G*S*-conjugates [40,41]. It is therefore reasonable to propose that the MRP/GS-X pump may export CP-PG G*S*-conjugates [25], thereby causing the modulation of the intracellular effects of CP-PG. PGA_1, which is a CP-PG, is also a potent inhibitor of methotrexate efflux via the MRP/GS-X pump by forming G*S*-conjugates that compete with methotrexate in tumor cells [42]. Moreover, human leukemia cells overexpressing the MRP/GS-X pump have been found to be resistant to treatment with a PGA derivative, while sensitivity to CP-PG treatment may vary enormously [43,44] and appears to be associated with MRP/GS-X pump activity [44]. As a rule, solid tumors show the highest detectable MRP/GS-X pump activity, comparable only to that found in hepatocytes, while leukemia cells and normal white blood cell-derived lines exhibit an extremely low level of activity [45]. Although rat resident macrophages exhibited low MRP/GS-X pump activity, Bacillus Calmette-Guérin (BCG)-activated macrophages enhanced transport activity conspicuously [45], thus suggesting that, under adequate stimuli (e.g. during the triggering of an immune response), macrophages are capable of providing a defense against electrophilic CP-PGs by extruding them through the MRP/GS-X pump. This is, however, not the case for lymphocytes; even after mitogenic stimulation, which induces dramatic alterations in lymphocyte activation and metabolism [46], MRP/GS-X pump activity in these cells was disproportionately low in relation to that found in other cell types, including other hematopoetic lines [45]. Hence, lymphocytes show a "functional absence" of MRP/GS-X pump activity, which may be insufficient to eliminate CP-PGs in a microenvironment where the CP-PG production may be high (e.g. in inflammation sites or the surroundings of tumor cells). Therefore, the above observations support the initial proposition that the accumulation of electrophilic CP-PGs in lymphoid tissues of Walker 256 tumor-bearing rats [12] may involve the absence of MRP/GS-X pump activity in lymphocytes. This contrasts with the high activity of the ATPase detected in many solid tumors, which may facilitate the elimination of CP-PGs. This statement is also in accord with the observation that, in Walker 256 tumor-bearing rats, the tumor tissue itself did not accumulate any appreciable level of CP-PGs [12]. Ultimately, deficient MRP/GS-X pump expression in lymphocytes of cancer-bearing subjects could have an adverse impact on host immune function (see Fig. 2), by allowing the accumulation of CP-PGs and immunosuppression, accelerated cancer cachexia, and death [12,45].

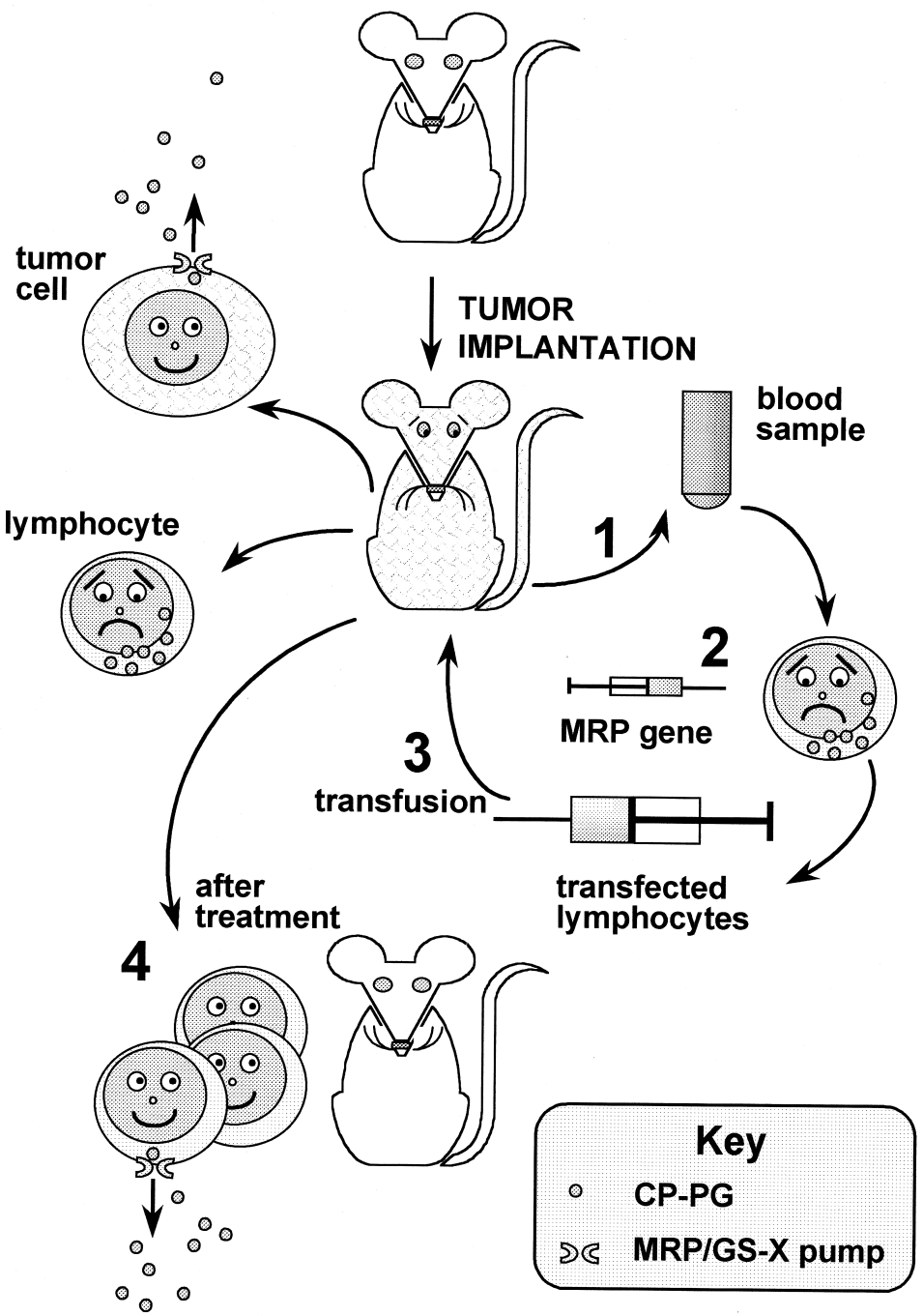

Fig. 3. Tentative MRP-transfected lymphocyte (MTL) therapy. Overproduction of the extremely cytotoxic and cytostatic CP-PGs may lead to host immunosuppression in the late stages of cancer. While tumor cells extrude CP-PGs towards the extracellular space as glutathione *S*-conjugates through the MRP/GS-X pump, lymphocytes do not exhibit any appreciable MRP/GS-X pump activity and thus accumulate CP-PGs. The possibility of the reversal of CP-PG-mediated immunosuppressive effects is under investigation, using Walker 256 tumor implantation in rats, as shown. After the tumor reaches rapid-growth phase, a blood sample is collected (**1**), lymphocytes are separated, transfected (**2**), and re-injected into the animal (**3**). It is expected that, after MTL therapy, lymphocytes may extrude CP-PGs through the MRP/GS-X pump. Without immunoblocking CP-PGs, lymphocytes may proliferate, restoring the immune system function and thus impairing tumor growth (**4**).

6. A clue for new strategies in cancer therapeutics and concluding remarks

MRP/GS-X pump expression may be an important agent in modulating the biological activity of CP-PGs by exporting them from the cells. The physiological role of such proteins may range from a protective function in chemical toxicity and oxidative stress [47] to the regulation of cellular activation and redox status [44,45] and cancer cell growth suppression [48]. Understanding the regulation of MRP/

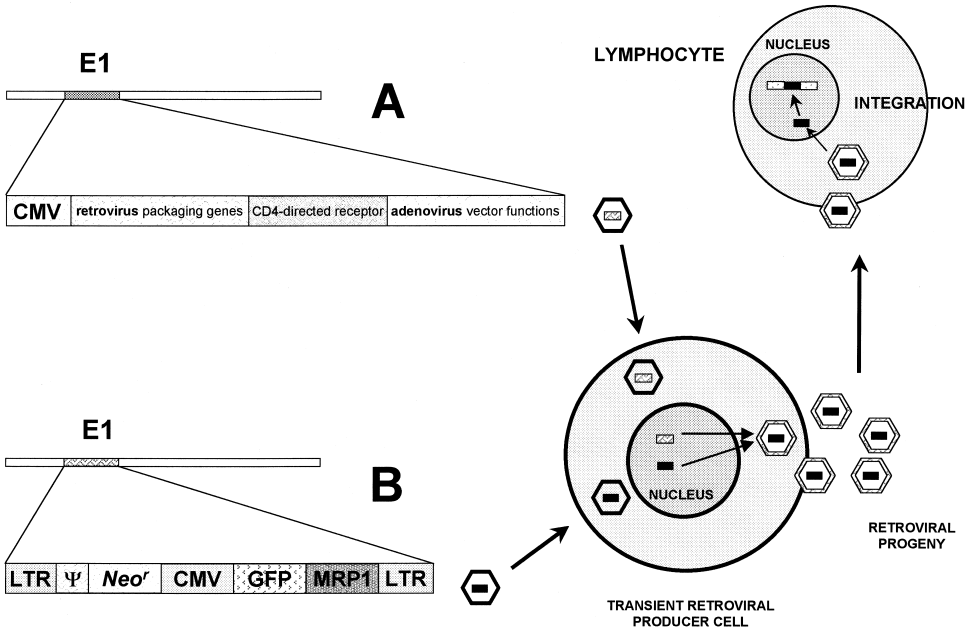

Fig. 4. Preparation and utilization of adenoviral-retroviral chimeras by incorporating specific DNA sequences into the Early (E1) region of an adenoviral vector. (A) DNA **retroviral** packaging function genes (essential protease *gag*, reverse transcriptase *pol*, and amphotropic envelope *env*) and $CD4^+$ T lymphocyte-directed receptor protein gene are incorporated into an **adenovirus** vector under the control of cytomegalovirus (**CMV**) intermediate/early enhancer-promoter. (B) An **adenoviral** vector with **retroviral** properties and carrying genes encoding for neomycin resistance (Neo^r), green fluorescent protein (GFP), and the MRP1/GS-X pump flanked by retroviral long terminal repeats (LTRs) and a packaging signal (Ψ). Transient retroviral producing target cells are converted by infection with a combination of both viral constructs. The chimeric retroviral progeny is then used to infect lymphocytes *in vivo* in order to achieve stable gene integration.

GS-X pump gene expression, therefore, will be an important step in unraveling new pathways of MDR and control of cell proliferation while providing useful information in the strategy of cancer chemotherapy practice. In parallel to the manipulation of MRP/GS-X pumps in tumor cells, however, the usage of MRP-transfected lymphocytes from peripheral blood of cancer patients for autologous transfusion purposes [see Fig. 3 for the tentative MRP-transfected lymphocyte (MTL) therapy proposed] is envisaged. This possibility is currently under investigation in this laboratory by using *MRP1* gene transfection and Walker 256 tumor-bearing rats as a model. Nevertheless, the number of lymphocytes that can be obtained through this process may be only a small part of the total lymphocyte bulk within the body, and the success of *MRP* gene transfection into these cells may occur only in a minor fraction of the lymphocytes collected. An alternative method of lymphocyte transfection has also been proposed, which is based on chimeric adenovirus-retrovirus vector approaches (for a review, see Ref. 49) used to improve the stable incorporation of heterologous genes into the host genome (gene integration) for long-term expression. Accordingly, we are testing for the preparation of adenoviral-retroviral chimeras by incorporating specific DNA sequences into the Early (E1) region of adenoviral vectors (Fig. 4) as described elsewhere [49]. DNA retroviral packaging function genes (e.g. essential protease *gag*, reverse transcriptase *pol*, and amphotropic envelope *env*) and the $CD4^+$ T lymphocyte-directed receptor protein gene are incorporated into an adenovirus vector under the control of cytomegalovirus (CMV) intermediate/early enhancer-promoter. Also, an adenoviral vector with retroviral properties has been prepared, with genes encoding for neomycin resistance (Neo^r), green fluorescent protein (GFP) and the MRP1/GS-X pump. These genes are flanked by retroviral long terminal repeats (LTRs) and a packaging signal (Ψ). Transient retroviral producing target cells will be converted by infection with a combination of both viral constructs. The chimeric retroviral progeny will then be used to infect lymphocytes *in vivo* in order to achieve stable gene integration and the capacity of transferring the MRP1/GS-X pump gene to the lymphocyte progeny.

Adenovirus-retrovirus chimeras take into account the advantageous features of both types of viruses such as high *in vivo* transfection, high titer production, and the capacity to infect both dividing and non-dividing cells. This will lead to chromosomal integration of the delivered gene(s) by the retroviral vectors and their long-term expression. Hence, the direct *in vivo* delivery of MRP/GS-X pump genes to the lymphocytes of cancer patients is expected in the near future. Inasmuch as host immunosuppression, due to the excessive accumulation of CP-PGs in immune tissues, is a major threat for the success of late-stage cancer therapeutics, the proposed strategy might improve the therapeutic outcome. By eliminating extremely cytotoxic and cytostatic high concentrations of CP-PGs within the lymphocytes through the MRP/GS-X pump, a novel clinical approach

becomes accessible in which the biological weapons against cancer cells will be the return of immune function to normal.

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