TUMOR ANGIOGENESIS AND INFLAMMATION AS THERAPEUTIC TARGETS OF RETINOBLASTOMA

Adriana Albini (a), Francesca Tosetti (b)
(a) Polo Scientifico e Tecnologico, IRCCS Multimedica, Milano
(b) Istituto nazionale per la ricerca sul cancro, Genova

Introduction

Retinoblastoma is the most common intraocular paediatric cancer, affecting 1 in 15000-20000 live births. The disease occurs in heritable form, usually bilateral, and sporadic or unilateral form. In both cases it arises from biallelic mutation of Rb gene. In bilateral form the first mutation occurs within the germline cells, while in the other form the mutational event is sporadic.

It is well established that stromal and immune cells release soluble factors that favor tumor progression by promoting the angiogenic switch through stimulation of dysregulated endothelial cell proliferation. Stromal factors further contribute to tumor growth and dissemination by inducing resistance to death and increased motility in tumor cells. Several lines of evidence indicate that inflammatory angiogenesis also promotes retinoblastoma progression. The main objectives of the project were:

1. to define the signaling pathways mediating the effects of immune and stromal-derived factors (VEGF, IGF-1) on retinoblastoma cell growth and resistance to cell death *in vitro*;
2. to define relevant signaling pathways activated in retinoblastoma cells that mediate tumor stimulated angiogenesis and inflammation *in vivo* (AKT/GSK3β, NFkB);
3. to perform in depth investigation of the biological effects of selected chemopreventive agents on retinoblastoma cells *in vitro*, in cell-free models *in vivo* (retinoblastoma conditioned medium-induced angiogenesis) and on retinoblastoma growth in cell-driven cancer models including xenotransplants and orthotopic models;
4. to study other ocular tumors (i.e., uveal melanoma) to develop intraocular xenograft murine model.

Results and methods

We studied the proapoptotic activity of the anticancer synthetic retinoid N-(4-hydroxyphenyl)retinamide (4HPR, fenretinide), a prototypical prooxidant anticancer agent, in retinoblastoma cells stimulated by the Insulin-like growth factor I (IGF-1). IGF-1 is a potent anti-apoptotic stromal-derived growth factor and sustains the autocrine growth of Y79 cells. 4HPR was used because it kills neurogenic tumor cells (neuroblastoma, retinoblastoma, glioblastoma, meningioma). 4HPR gave encouraging results in a phase I trial in children affected by neuroblastoma. We observed that 4HPR reduces retinoblastoma tumor growth *in vivo* by inducing reactive oxygen species and necrosis-like cell death in Y79 cells. As a consequence of impaired redox equilibrium, 4HPR disrupts energy balance, as indicated by ATP depletion and loss of mitochondrial membrane potential. 4HPR inhibited AKT and mTOR phosphorylation induced by IGF-1 in Y79 cells. To investigate the interference of 4HPR with IGF-1-mediated survival signaling at molecular level, we analyzed the down-stream target and

AKT effector GSK3β, a multifunctional kinase regulating cell survival, glucose metabolism and the beta-catenin/Wnt pathway. GSK3 β is inactivated by phosphorylation at Ser9 by AKT and other kinases and is normally active (unphosphorylated) in unstimulated cells. 4HPR sustained GSK3β phosphorylation at Ser9 in IGF1-1-treated retinoblastoma cells. Treatment with 4HPR alone induced time-dependent GSK3β phosphorylation that was coincident with cleavage of poly (ADP-ribose) polymerase (PARP). Perturbation of IGF signaling by 4HPR correlated with the lack of effect of IGF-1 on PARP cleavage and DNA fragmentation induced by 4HPR at 24 h. Time-dependent ROS production by 4HPR preceded activation of antioxidant enzymes, GSK3 phosphorylation and PARP cleavage.

In order to characterize the pro-angiogenic potential of retinoblastoma tumors, we preliminary investigated the angiogenic molecules released by retinoblastoma cells. The Y79 cell line was used to analyze the contribution of the Insulin-like growth factor I (IGF-1)/IGF-1 receptor (IGF-1R) system. IGF-1 is a survival factor for retinal cells, a potent proangiogenic factor and promote angiogenesis by inducing hypoxia inducible factor-1alfa (HIF-1alfa) activation. We suspected that IGF-1 released by Y79 cells could substantially contribute to retinoblastoma -derived angiogenesis. In fact, IGF-1 (10 ng/mL) added to the matrigel plug effectively induced angiogenesis, that was inhibited by 4HPR. The serum-free conditioned medium (CM) from a 48h culture was concentrated and injected subcutaneously into the flanks of C57/bl6 male mice with matrigel. The treatments included the CM from Y79 cells exposed for 48h to the IGF-1R inhibitor H1356 and samples containing H1356 directly added into the matrigel plug along with the CM from untreated cells. The results obtained confirm, as previously shown 1, that Y79 cells release soluble factors capable of inducing a potent angiogenic response in the matrigel plugs, as determined by the quantification of hemoglobin content. The histologic examination of hematoxylin- and eosin-stained sections of the experimental tumors revealed the formation of large vessels. The obtained data suggest that retinoblastoma cells stimulate angiogenesis through the production of soluble factors strongly dependent on the IGF-1R, probably activated by autocrine IGF-1. We observed that retinoblastoma cells express high basal levels of HIF-1alfa. We are now investigating whether constitutive HIF-1alfa expression, which is characteristic of embrionic stem cells, could be related to the staminal properties of retinoblastoma cells. Tumor regression apparently correlates with enhanced expression of HIF-1alfa in necrotic areas, where the inflammatory infiltrate does not seem to increase. These results are in line with a proapoptotic role of HIF-1 that has been documented in several tumor model systems.

We observed that retinoblastoma cells basally produce large amounts of reactive oxygen species 1, 2, as compared with other tumor cell types including Rb-/- melanoma, suggesting that constitutive oxidative stress is of physiological importance in this tumor. Following the idea that elevated oxidative stress in tumor cells could be exploited to selectively induce cell death by prooxidant drugs, we further investigated the apoptotic effects of diverse prooxidants in retinoblastoma. This therapeutical approach received great attention recently, also in the case of retinoblastoma. We compared the activity of 4HPR with arsenic trioxide and the isothiocyanate PEITC in Y79 and Weri-Rb1 cells. We found remarkable differences between the two cell lines in the biological response to anticancer prooxidants. Retinoblastoma cells can activate an antioxidant defense response involving increased expression of the cytoprotective molecules heme oxygenase (HO-1), superoxide dismutase (SOD) and glucose 6-phosphate dehydrogenase (G6PD). The antioxidant response also activates redox cycling of glutathione (GSH). This response is regulated by survival signaling mediated by AKT, GSK3 and ERK. Flow cytometry analysis with the fluorescent dye monochlorobimane revealed the existence of subpopulations of Y79 and Weri-R1 cells containing different levels of glutathione, correlating with different basal level of oxidative stress as indicated by H2O2 content. We selected Y79 and Weri-Rb1

cells resistant to 4HPR. The resistant cells show basal elevated GSH and ROS levels and GSK3β phosphorylation. In conclusion, we found that while transient GSKβ and ERK1/2 phosphorylation is activated by mild oxidative stress, a cytoprotective response is elicited in tumor cells. This mechanism could support the development of drug resistance to anticancer agents. However, persistent GSK3 and ERK1/2 activation can lead tumor cells to death due to activation of a futile antioxidant redox cycle and energy depletion.

We adopted a gene transduction approach using a potent TH1 cytokine with strong anti-angiogenic activity, Interleukin-12 (IL-12). The 99E1, a cell line derived from a choroidal/retinal pigmented epithelial ocular tumour that arose in a transgenic FVB/N mouse bearing the SV40 oncogene, were trasfected with murine IL-12 expression construct. After transfection, the 99E1 cells were selected for transgene expression using Geneticin G-418 sulphate and the resistant clones pooled into two different pools of stably transfected cells. The enhanced levels of IL-12 mRNA expression and protein production were confirmed by RT-PCR and ELISA, respectively. About 6-7 week old female nude mice were inoculated, s.c. and intraocular transplantation, with 99E1 wild type, 99E1 null vector or IL-12 trasfected 99E1 cells.

Orthotopic intraocular injection of control cells led to invasive tumors that destroyed ocular architecture whereas the IL-12 transduced cells rarely formed tumours. When intraocular tumor invaded the eye anterior chamber the tissues were removed for histological analysis. Samples were fixed in formalin, embedded in paraffin using standard procedures, and 3 μm sections were rehydrated and stained with either hematoxylin and eosin or processed for immuno-histochemistry by standard procedures to analyze VEGF and asilo-GM1 expression. Histological analysis revealed highly invasive and angiogenic tumor growth in the controls and poorly vascularized tumors in the presence of IL-12. The tumour repression effect could be reproduced by a systemic anti-angiogenic effect, where controlateral injection of IL-12 expressing cells strongly repressed growth of tumors formed by parental 99E1 cells. This was associated with significantly lowered tumor vessel densities, a trend towards lower VEGF levels in the lesion, and significantly decreased NK cells in the parental tumors exposed to systemic IL-12. Taken together, these data suggest that IL-12 gene transfer can provide anti-angiogenic effects without toxicity and may be particularly suited for therapy of vascularized ocular tumors.

Conclusion

The study of the action of anticancer prooxidant phytochemicals or their synthetic derivatives in retinoblastoma offered us the opportunity to formulate a hypothesis on the apparently paradoxical effects of these compounds. In which way the cytoprotective and normalizing effect in neurological, cardiovascular and metabolic disorders can match the antitumor effect based on induction of apoptosis or other forms of cell death? The explanation is apparently simpler in a cancer chemopreventive setting: redox active phytochemicals that arose during evolution as toxic pesticides in plants, reinforce the stress defense by limiting genotoxicity, mutagenicity and by improving the antioxidant capacity of the cells. But what about the cytotoxic effect on established tumor cells? We hypothesize that the intolerance to drug-induced oxidative stress could be due to the particular bioenergetic requirements of tumor cells which rely on glycolysis for energy production even in the presence of normal oxygen tension (Warburg effect). Although in tumor cells an intact respiratory chain is structurally preserved, its functionality is often reduced. Interestingly, forcing a diversion on mitochondrial respiration for energy production can kill glycolytic tumor cells. The constitutive oxidative

stress found in tumor cells has been related to the overactivation and overexpression of oncogenes including ras and bcr-abl. The requirement for an increased production of reducing equivalents (NADPH) to counterbalance the constitutive oxidative stress could justify at least in part the glycolytic switch for energy production.

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