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Invited Review

Apoptosis in cancer: therapeutic implications

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Summary. This review outlines the principal limitations of the mechanisms of active cell death (ACD, apoptosis) as the basis of tumorigenesis and the rationale of almost all therapies of malignancy. The concentration of cancer therapy in the directon of ACD induction is presented as both the result of progressive understanding of the mechanisms of apoptosis and that of the favourable tumor environment for ACD signal transmission. The latter property induces the by-stander killing of cancer cells, a fundamental mechanism because efficiency of all known methods of cancer treatment is far below 100%. Finally, recent results and hypotheses regarding cancer gene therapy based on final inductors of apoptosis and endogeneous ACD inhibitors in tumors are evaluated.

Key words: Cancer, Apoptosis, Bax, Caspases, Alpha2 macroglobulin, By-stander killing

Introduction: Cell loss factor in tumors

In current years, the simplistic view considering cancer the result of enhanced cell proliferation is being quickly replaced by the broader image of malignancy as consisting in an anomalous accumulation of cells, implying that the comparative rates of cell proliferation and cell death determine how fast a tumor grows. Actually, there are cancer cells which divide more slowly than their benign origin cells, being malignant only due to delayed cell death (Green et al., 1994; Kerr et al., , 1994).

However, the balance between cell gain through mitosis and cell loss in neoplasia is tight, because cell loss is large. Cell production can be obtained either from the labeling index (i.e. the proportion of cells incorporating labeled thymidine after a labeling pulse) or from the mitotic index (i.e. the percentage of mitotic cells). These evaluations allow the calculation of the potential doubling time (Tp, i.e. the time in which the cell number would have doubled had there been no cell loss). The actual doubling time (Ta) is given by direct measurements of tumors. These two parameters permit the calculation of the cell loss factor (CLF = 1 - Tp/Ta). With no cell loss, CLF is zero; it tends to unity if loss is so extensive that Ta becomes very large. One cardinal characteristic of virtually all tumors which have been submitted to this type of measurements is that cell loss factors are huge. Even in transplantable animal tumors selected for rapid growth CLF exceeds 0.6. In aggressive human cancers values are still higher, such as 0.73 in melanoma, 0.96 in colorectal carcinoma and range from 0.71 to 0.99 in pulmonary tumors of different histological types. Moreover, the cell loss factor augments during tumor enlargement. Three main types of cell loss occur in tumors: exfoliation, metastasis and cell death, with the latter factor accounting for the majority of cell elimination (Kerr and Lamb, 1984; Steel, 1967, 1968; Wyllie, 1985; Granville et al., 1998).

Malignant tumors frequently contain necrosis. Equivalent to passive cell death, necrosis is the result of hypoxia since it occurs at a distance from blood vessels which is quite constant for individual tumors and its extension is influenced by the level of oxygenation of the blood (Schatten, 1962). Though conspicuous histologically, necrosis does not satisfactorily account for most of the cell losses predicted on the basis of CLF since important loss factors can occur in turnors with high mitotic indices and no necrosis. It is therefore likely that apoptosis is responsible for an important part of cell death in cancer. The term "apoptosis" was coined to describe genetically driven, active, cell death (ACD). And indeed, apoptosis exists in all malignant lesions. The factors responsible for the universal spontaneous occurrence of ACD in tumors are not unequivocally established. They presumably belong to two classes: 1) cell population regulatory mechanisms originating in the host organism, the so-called "social controls on cell death"; and 2) apoptosis initiated by processes intrinsic to malignant cells (Schatten, 1962; Kerr and Searle, 1972; Kerr et al., 1972, 1994; Wyllie et al., 1980; Wyllie, 1985; Sarraf and Bowen, 1988; Raff, 1992; Staunton and Gaffney, 1995; Granville et al., 1998; Soini et al., 1998).

Due to the relative rapidity of the phenomenon (10 minutes to 40 hours for chromatin condensation and cell fragmentation, 3 hours for apoptotic-body phagocytosis and digestion), strong ACD-induced tissular decrease is

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often based on less than 5% of the cell population of histologically visible apoptotic cells and bodies. This presumably precluded accurate determinations of ACD contribution to tumor cell deletion. One such quantification showed that apoptosis accounts for around 7% of total CLF in murine sarcomas (Sarraf and Bowen, 1986) but this figure may be reasonably held as a minimum because tumor ACD occurs unevenly (with bursts) and its incidence strongly depends on the histological type of tumors. Moreover, in contrast with necrosis, apoptosis is generally of a non-inflammatory nature. Finally, apoptosis is inversely related to necrosis, i.e. tumors with high apoptotic rates grow slowly and show little necrosis while those with low ACD rates expand quickly and contain much necrosis, a situation corresponding to the familiar counterintuitive notion of pathologists that tumors with a large proportion of necrosis have a bad prognosis (Wyllie, 1995; Sarraf and Bowen, 1988; Bursch et al., 1990; Arends et al., 1994; Staunton and Gaffney, 1995; Collins et al., 1997; Messam and Pittman, 1998; Soini et al., 1998).

Yet, apoptosis quantitation in tumors is quickly improving due to recent technical evolutions (Soini et al., 1998). Since most human tumors are mainly accessible only as fixed, paraffin-embedded samples, one important progression concerns the in situ detection of apoptotic cells. In the absence of a universal specific molecular tracer of apoptosis, structural DNA alterations provide the basis of labeling systems: double-strand fragmentation for TUNEL (terminal transferase-mediated dUTP nick end-labeling); denaturation for poly (A) in situ hybridization; and immunogenicity of single strand DNA, all methods which imply limited specificity due to the unavoidable presence of DNA breaks in virtually all cells. Thus, TUNEL, the most used of the above methods, has been restrained to a narrow spectrum of sample conditions which has limited, in particular, retrospective surveys. In the apoptotic nucleus two main obstacles intervene between TUNEL reagents and their targets: DNA hypercondensation and proteins around DNA. The former increases in the course of apoptosis and both are worsened by crosslinking and precipitating fixatives. This points out that TUNEL is an ambitious approach whose targets, apoptotic DNA breaks, are less accessible than breaks occuring in non-apoptotic less compacted DNA. However, TUNEL has an advantage: the far greater degree of apoptotic DNA fragmentation. This made it possible to reach a frank differential staining between apoptotic and non-apoptotic DNA via adapted pretreatments, i.e. the combination of proteolysis and microwaves. Moreover, inasmuch as labeling is considered on an all - or - nothing basis, with every stained nucleus, weakly or strongly, scored, quantification is not impaired, provided it is ascertained by morphological criteria (Sträter et al., 1995; Negoescu et al., 1996b; Labat-Moleur et al., 1998).

The above data make plausible a double hypothetical suggestion: that the limit between tumor growth and elimination could be crossed by a relatively small augmentation in the rate of cell death. And that the ACD moiety of tumor cell death, by its intrinsic property of being highly regulated and inducible, could provide the therapeutic way to increase cell losses enough to displace tumors from growth to regression.

This hypothesis has received broad confirmation. An exponentially growing body of reports has created, during the last 15 years, the general concept that all non surgical modalities of cancer therapy act primarily via the induction of apoptosis: radiotherapy, thermotherapy, chemotherapy, immunotherapy and hormone ablation (Lennon et al., 1991; Tenniswood et al., 1992; Green et al., 1994; McDonnell et al., 1995; Hannun, 1997). A general property which provides the therapeutic window of opportunity is the apoptosis threshold which is higher in normal as opposed to cancer cells. Thus, normal tissues may repair the damage induced by therapy while tumor cells undergo ACD (Fisher, 1994; Green, 1998).

Therefore, we feel that research in the area of cancer therapy is concentrating onto the induction of apoptosis. However, the actual level of this focusing process has not yet received, to our knowledge, an up-to-date global critical evaluation, except for gene therapy (Favrot et al., 1998). Using our practice and that of others in the field we consider useful to compensate this lack.

Main molecular tracks to apoptosis in cancer cells

The study of gene regulation in malignant cells indicates that cancer is predominantly a genetic disease which develops through a multistep process involving oncogenes and tumor suppressor genes. The former derive from genes, named protooncogenes, that participate in normal functions of the cell such as signal transduction, growth regulation or transcription and a single abnormal allele in these genes is required for malignant transformation. Heterozygous modifications of protooncogenes which confer the transforming function include mutations, translocations, re-arrangements and amplifications. Tumor suppressor genes (antioncogenes) normally inhibit tumor development by regulation of DNA transcription, cell proliferation or ACD; monozygous loss of function of antioncogenes through mutations or deletions is involved in the development of a high proportion of human cancers (Cory and Adams, 1998; Evan and Littlewood, 1998).

The most important among the multiple genetic events associated with tumor development are activation of oncogenes such as myc family members (Cory and Adams, 1998; Evan and Littlewood, 1998) and Bcl_2 (Kroemer, 1997; Adams and Cory, 1998; Chao and Korsmeyer, 1998) and inactivation of tumor suppressor genes including Rb (Cory and Adams, 1998), p53 (abnormal in more than half of all malignant cell types (Ko and Prives, 1996)), and Bax (Yin et al., 1997; Brady and Gil-Gomez, 1998), all being genes involved in apoptosis. Indeed, c-Myc signals cellular proliferation. However, in cells lacking other specific mitogenic

stimuli deregulated c-Myc induces ACD (Evan et al., 1992). Analogously, the Rb protein can be either an apoptosis suppressor or inducer. Finally, ACD regulation is the central function of p53 and Bcl_2 gene family members, among which Bax plays a central role (Kroemer, 1997; Adams and Cory, 1998; Chao and Kormeyer, 1998; Evan and Littlewood, 1998).

In the control of apoptosis, a central position is currently ascribed to the members of the Bcl_2 gene family (Oltvai et al., 1993; Yang and Kormeyer, 1996; Wang and Reed, 1998). The Bcl_2 gene was discovered in a B-cell lymphoma where a chromosomal translocation t (14;18) moves the Bcl_2 gene into juxtaposition with transcriptional enhancer elements of the immunoglobulin heavy chain locus (Tsujimoto et al., 1985). In solid tumors, trans-regulatory mechanisms appear to be responsible for the high levels of Bcl_2 protein production (Hockenbery, 1994; Brambilla et al., 1996; Yang and Korsmeyer, 1996). One of the trans-regulators of Bcl_2 is the tumor suppressor protein P53. Negative responsive elements have been found in the 5' untranslated regions of the Bcl_2 gene through which wild type (wt), but not mutant P53, is able to mediate repression of the Bcl_2 gene. P53 mutations could thus result in elevated production of Bcl_2 at least in some tissues. In addition, wt P53 was found to strongly induce the expression of a homolog of Bcl_2 termed bax and a P53 binding site was identified in the bax gene promoter (Miyashita et al., 1994a,b,; Miyashita and Reed, 1995). The Bax protein, sharing 21% homology with bcl_2 protein, can be considered as the main effector of apoptosis (Yin et al., 1997; Brady and Gil-Gomez, 1998). Its function in active cell death as a dimer Bax-Bax can be opposed by hetero-dimerization with Bcl_2 (Bax-Bcl_2). The ratio of the two proteins would determine the predominant type of dimer (the "rheostat model") (Korsmeyer et al., 1993). Thus, p53 appears to be an important regulator of apoptosis through the Bax-Bcl_2 balance, and p53 mutation, or any other type of p53 inactivation, could be responsible for abrogation of cell death through Bax/Bcl_2 dysbalance (Korsmeyer et al., 1993; Brambilla et al., 1996; Yin et al., 1997; Evan and Littlewood, 1998; Wang and Reed, 1998; Soengas et al., 1999). Alternatively, lack of Bax protein in cancer cells can be the result of mutations in the bax gene (Rampino et al., 1997).

Thus, the situation is special for Bax, because this molecule is placed at the final part of the apoptotic pathway. A model has emerged giving Bax the role of the penultimate effector of ACD, directly before the final step of apoptosis induction (the "execution" (Martin and Green, 1995), represented by caspase proteolysis (Alnemri et al., 1996; Green, 1998; Shaham, 1998)). Caspases are triggered by the Bax-Bax homodimer and blocked by Bax-bcl_2 and bcl_2-bcl_2 heterodimers (Chinnaiyan et al., 1996; Perry et al., 1997; Adams and Cory, 1998). Yet, matters are far from so simple. Indeed, Bax can induce apoptosis independently of the dimerization process (Simonian et al., 1996) and even without caspase activation (Xiang et al., 1996). Alternatively, caspases are capable of self-induction (Thornberry and Lazebnik, 1998), and can also be activated by FADD (Fas - associated protein with death domain), via FLICE (FADD-like ICE, caspase 8), without mediation by Bax homodimers (Strasser et al., 1995; Nagata, 1997; Ashkenazi and Dixit, 1998; Schultze-Osthoff et al., 1998). Moreover, caspases can convert Bcl_2 to a Bax-like death effector (Cheng et al., 1997) and Bcl_2 prevents caspase-independent cell death (Okuno et al., 1998).

Cancer therapy concentrates within apoptosis (Fig. 1)

Most features of cancer cell biology have been tentatively exploited for therapy. This generated rather diverse approaches, according to various treatment techniques. Yet, as shown below, the end-point of virtually all these methods is the induction of apoptosis.

a) Radiotherapy and thermotherapy

Ionizing radiation-based elimination of tumor cells consists in the initiation of apoptosis (Stephens et al., 1991, 1993; Foster et al., 1992; Meyn et al., 1993; Radford et al., 1994). While less investigated than radiotherapy, mild heating of tumor cells (therapeutic hyperthermia) achieves constantly an analogous ACD initiation (Barry et al., 1990; Harmon et al., 1990, 1991; Takano et al., 1991). The form of cell death changes from apoptosis to necrosis above a critical heat load (Harmon et al., 1990).

Radiation-induced ACD is inhibited by Bcl_2 and BclXL, two major members of the Bcl_2 family of apoptosis regulators (Han et al., 1995). Bax transfection sensitizes breast cancer cell to radiation-induced ACD. The dependence upon P53 function is not constant. Indeed, wild-type (wt) p53 transfection strongly augments the ACD-based cytotoxicity of ionizing

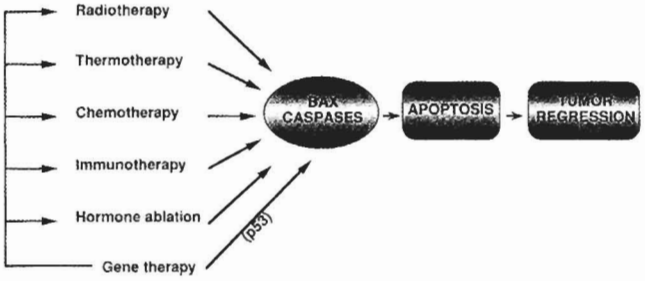

Fig. 1 Apoptosis as the final common way for cancer therapy. Tumor regression is achieved by various cancer therapies, which all unite in initiating active cell death (apoptosis). Gene therapy attempts to enhance the other methods of treatment and also usines directly the apoptotic pathway. In this general landscape, therapy focused on final inductors of apoptosis (the main being Bax and proteases termed caspases) appears as more promising than classically-used, upstream effectors such as p53.

radiation, as demonstrated with colon carcinoma cells. Interestingly, wt P53 did not have by itself a cytotoxic effect on these cells. On the other hand, radiaton-induced apoptosis in human colorectal carcinoma cells can occur in the absence of wt P53 (Bracey et al., 1995). Furthermore, in vivo, combined p53-radiation therapy reduced colorectal carcinoma growth within nude mice more efficiently than each of the two methods performed alone (Gjerset et al., 1995; Spitz et al., 1996; Yang et al., 1996a). Finally, another apoptotic pathway, distinct from that regulated by P53 but also inhibitable by Bcl_2-related molecules, is activated by the anti-oncogenic interferon regulatory factor (IRF1). Overexpression of this gene augments radiation sensitivity (Tanaka et al., 1994; McBride and Dougherty, 1995; Tamura et al., 1995).

Thus, the treatment benefit to be derived from combining gene therapy with radiotherapy is twofold: increasing radiosensitivity via exogeneous-gene expression and, conversely, augmentation of the effectiveness of gene therapy strategies by radiation (McBride and Dougherty, 1995; Bourhis et al., 1996). The former direction is best represented by radioimmunotherapy (RAIT), an approach based upon the idea that a radionuclide-carrying targeting molecule (e.g. antibody) can selectively deliver ionizing radiation to tumor sites. During the last decade, the success of this strategy was limited to malignant lymphomas (Macklis et al., 1992; Raben et al., 1996). One essential cause of these disappointing results is low intratumoral expression of the target antigen. Thus, gene transfer methods have been tried to achieve increased levels of tumor cell expression of the target antigen. Raben et al. (1996) showed in vitro that human glioma cells that do not express carcinoembryonic antigen (CEA) can be transduced with an adenovirus encoding human CEA which makes them a selective target for a ^{131}I-coupled monoclonal CEA antibody. In vivo, radiolabeled antibody ligation was achieved by in situ tumor transduction with the same vector. Identical experiments were performed by the same investigators on ovarian cancer, non small cell lung cancer and breast cancer cells (Buchsbaum et al., 1996).

Within the opposite approach, i.e. using radiation to enhance other methods of cancer gene therapy, two strategies must be mentioned. In immunotherapy, spatial and temporal control of tumor necrosis factor alpha (TNFα) expression by a radiation-inducible promoter/enhancer proved able to improve the immune stimulation and induction of cell death, a result related to the property of TNFα to enhance radiosensitivity (Sersa et al., 1988; Weichselbaum et al., 1992, 1994; Hallahan et al., 1995; Seung et al., 1995). Transduced herpes simplex virus thymidine kinase (HSV-TK)-based chemotherapy (see below) benefited from both enhanced expression of HSV-TK following ionizing radiation (Boothman et al., 1994) and the improvement of radiation response of cells transduced with this suicide gene (Kim et al., 1994, 1995).

b) Chemotherapy

It is accepted that the vast majority of chemotherapeutic agents act via apoptosis induction (Searle et al., 1975; Dive snf Hickman, 1991; Marks and Fox, 1991; Hickman, 1992; Sen and d'Incalci, 1992; Hannun, 1997). Drug-induced ACD essentially relies upon p53 function, though alternative ways most probably act in p53-deficient cells. Thus, in vitro expression of wt p53 into cancer cells powerfully enhanced their sensitivity to apoptosis induced by drugs, such as platinum salts and topoisomerase II inhibitors. These results were essentially obtained for glioblastoma, liver and colon cancer cells (Gjerset et al., 1995; Xu et al., 1996; Yang et al., 1996a). Accordingly, mdm2 antisense oligonucleotides, by liberating p53 activity, increased the in vitro susceptibility of a glioblastoma cell line to cisplatin-induced apoptosis (Kondo et al., 1995). In vivo, experiments performed in nude mice for lung cancer cells indicated that cisplatin plus p53 gene transfer strategy yielded significantly greater apoptosis and tumor growth retardation than wt p53 gene transfer alone. To underline the complexity of the mechanisms involved in p53-mediated potentiation of chemotherapy, in vitro incubation of acute myelogenous leukemia cells (wt p53) with p53 antisense oligonucleotides resulted in a 90% reduction of the cell population. When the surviving cells were replated in the absence of the oligonucleotides, a continuing decline in cell number was recorded. Since preclinical trials demonstrated a very low toxicity of these oligonucleotides, a phase I clinical trial was conducted in 16 patients. Though no clinical complete response was obtained, leukemic cell growth was reduced and their sensitivity to apoptosis-inducing agents augmented. Measurement of p53 expression demonstrated a transient decline during the initial treatment with the antisense construct, followed by a fourfold increase. This led the authors to hypothesize that the transient inability of p53 to respond to the stimulatory messages given by drug-induced DNA damage eventually results in p53 overexpression driving cells into ACD (Bayever et al., 1994; Bishop et al., 1996).

The ACD basis of chemotherapy provided fruitful evolutions in connected fields, the main of these being protocols making use of drug-sensitivity genes and drug resistance genes.

Drug sensitivity genes (cell suicide genes) are designed to create artificial differences between normal and malignant cells by making the latter sensitive to drugs inocuous to the former. Suicide genes achieve their function by encoding viral or bacterial enzymes which convert inactive forms of a drug (termed prodrugs) into cytotoxic drugs. The approach is sometimes termed gene-directed enzyme prodrug therapy (GDEPT). From in vivo studies it was estimated that, for significant effect, the drug must be at least 100-fold more cytotoxic than the prodrug (Moolten, 1994; Connors, 1995). The original theory of GDEPT was to preemptively generate

a mosaicism within individuals so that scattered cells differ in terms of what drugs they are sensitive to. If a tumor later arises, therapy is directed at whatever sensitivity the tumor clone has, while the host is protected by the majority of the cells in the mosaic normal population which do not share that sensitivity (Borrelli et al., 1988; Moolten and Wells, 1990; Moolten et al., 1990; Moolten, 1994, 1986). This approach appeared as impractical and thus systems were devised to deliver prodrug-processing enzymes to preexisting tumors (Freeman et al., 1993).

Different such suicide genes have been tried, as those coding for: cytosine deaminase (which converts 5-fluorocytosine to the cytotoxic 5-fluorouracyl (Huber et al., 1991; Harris et al., 1994; Mullen et al., 1994; Hirschowitz et al., 1995)), xanthine-guanine phosphoribosyl transferase (encoded by the gpt gene and transforming 6-thioxanthine to 6-thioguanosine monophosphate which impairs DNA synthesis (Mroz and Moolten, 1993)), purine nucleoside phosphorylase (encoded by the DeoD gene and catalysing the conversion of deoxyadenosine analogs to toxic adenine analogs, (Hughes et al., 1995)), cytochrome P450 2B1 (which produces alkylating metabolites from oxazaphosphorines (cyclophosphamide or ifosfamide), (Osaki et al., 1994; Chen and Waxman, 1995), and varicella-zoster virus thymidine kinase (VZV-TK, which converts 6-methoxypurine arabinonucleoside (araM) to toxic adenine arabinonucleoside triphosphate (araATP) (Huber et al., 1993)). However, the main suicide gene in use is that coding for herpes simplex virus thymidine kinase (HSV-TK) which converts the antiviral agent acyclovir (Furman et al., 1980; Hasegawa et al., 1993; Shewach et al., 1994) and its mostly used derivative ganciclovir (GCV) to their monophosphate forms, an ability lacking to mammalian kinases. Subsequent conversion to the diphosphate (by HSV-TK or cellular kinases), followed by addition of one more phosphate groups by cellular kinases yields a triphosphate which replaces deoxyguanosine triphosphate, normally used for DNA synthesis. This blocks DNA polymerase from elongating DNA beyond the point of GCV incorporation resulting in a thousandfold higher cytotoxicity than performed by the prodrug (Moolten, 1994; Connors, 1995).

The HSV-TK gene was transfected either by directly injecting engineered adeno- or retroviruses (Abe et al., 1993; Caruso et al., 1993; Smythe et al., 1994; Hwang et al., 1995; O'Malley et al., 1995; Rosenfeld et al., 1995; Yoshida et al., 1995; Elshami et al., 1996) or using ex-vivo transfected producer cell lines (fibroblasts or previously excised cells) introduced into the tumor to provide a replenishable supply of viruses over time (Ezzendine et al., 1991; Plautz et al., 1991; Culver et al., 1992; Molten et al., 1992; Freeman et al., 1993; Ram et al., 1993; Barba et al., 1994; Iziquierdo et al., 1995; Yee et al., 1996). A variant of the method is to place the HSV-TK gene under the control of a promoter restricted to cancer cells, such as that of alpha-fetoprotein in hepatomas (Macri and Gordon, 1994; Ido et al., 1995), carcinoembryonic antigen or myc in lung cancer cells (Osaki et al., 1994; Kumagai et al., 1996) or DF3 mucin-like glycoprotein in breast carcinoma cells (Manome et al., 1994).

These various approaches allowed a large panel of malignant cells to be included in in vitro, animal or clinical protocols using HSV-TK. The most salient include tumor cells of the following origins: neural (Ezzedine et al., 1991; Culver et al., 1992; Takamiya et al., 1992; Ram et al., 1993, 1994; Barba et al., 1994; Chen et al., 1994; Shewach et al., 1994; Wu et al., 1994; Iziquierdo et al., 1995; Pyles et al., 1997), liver (Caruso et al., 1993; Macri and Gordon, 1994; Ido et al., 1995), digestive tract (Chen and Waxman, 1995; Kaneko and Tsukamoto, 1995; O'Malley et al., 1995; Yoshida et al., 1995), lymphomas (Moolten et al., 1990b; Abe et al., 1993), ovary (Freeman et al., 1995; Rosenfeld et al., 1995; Alvarez and Curiel, 1997), breast (Balzarini et al., 1985; Manome et al., 1994; Yee et al., 1996), mesothelium (Smythe et al., 1994; Hwang et al., 1995; Elshami et al., 1996), lung (Hasegawa et al., 1993; Osaki et al., 1994; Smythe et al., 1994; Hwang et al., 1995; Kumagai et al., 1996; Zhang et al., 1997), melanoma and fibrosarcoma (Bi et al., 1993; Vile and Russell, 1993, 1994; Wu et al., 1994).

The efficiency of the HSV-TK/GCV method depends on the cell type, being, for example, around 20 times stronger in mammary cancer cells than in lymphoma cells (Beck et al., 1995). These differences may be connected to the fact that suicide-gene cytotoxicity is subject to various regulations since it mainly consists of apoptosis induction (Samejima and Meruelo, 1995; Hamel et al., 1996) together with certain levels of necrosis (Chen and Waxman, 1995; Kaneko and Tsukamoto, 1995). ACD induction is tightly connected to the well-known ability of suicide genes to kill pre-existing unmodified cells located in the neighbourhood of transfected cells (the by-stander effect (Culver et al., 1992; Freeman et al., 1993). By-stander apoptosis is of paramount importance for cancer gene therapy and is not restricted to suicide genes, as shown below.

Also connected to apoptosis is the development of drug-resistance gene therapy, an approach that tends to make chemotherapeutic dose escalation protocols feasible by limiting cytotoxicity in bone marrow cells (Whartenby et al., 1995). This was attempted in animal experiments either by transfection of the multiple-drug resistance gene (MDR1) (Guild et al., 1988; McLachlin et al., 1990; Mickish et al., 1992; Sorrentino et al., 1992) which produces the P-glycoprotein cellular efflux pump or of genes encoding for more specific protectors against cytotoxic drugs like O-methylguanidine-DNA-methyltransferase (MGMT) (Moritz et al., 1995), against nitrosourea or dihydrofolate reductase (DHFR) and against methotrexate (May et al., 1995) or trimetrexate (Spencer et al., 1996). This methodology tends to limit ACD induction in bone marrow cells. Alternatively, enforced expression of anti-apoptotic genes can be used to diminish ACD-based myelosuppression during cancer

chemotherapy. Indeed, in vitro experiments demonstrated that Bcl_2-transduced murine bone marrow cells resist to apoptosis induced by both topoisomerase I and II inhibitor cytotoxic drugs (Kondo et al., 1994). Similarly, in vitro experiments demonstrated that $bclX_L$ is a strong inhibitor of chemotherapy-induced apoptosis. What is more, this property spans over a wide variety of cells and anticancer agents, its expression conferring a multidrug resistance phenotype (Dole et al., 1995; Minn et al., 1995).

c) Immunotherapy

Cancer cells generally evade immune surveillance since they often have too few non-self features and through the production of immunosuppressive agents. Tumor destruction is essentially mediated by T-cytotoxic (CD8+) lymphocytes (CTL) and by natural killer (NK) cells, the latter acting as lymphokine-activated killer (LAK) cells. CTL and LAK are grouped under the term tumor infiltrating lymphocytes (TIL) (Townsend and Allison, 1993; Nabel et al., 1994; Culver et al., 1995). For an effective response at least three signals are necessary. Firstly, immunogenic antigens must be presented by tumor cells. Secondly, the CD8 T cell receptor must be occupied by a major histocompatibility complex (MHC) class I peptide complex. Finally, helper (CD4+) T cells must be activated to provide stimulating cytokines to CTL and LAK (Culver et al., 1995; Golstein et al., 1995).

The first of these requirements formed the basis of polynucleotide vaccination using a plasmid DNA encoding for human carcinoembryonic antigen (CEA, the most extensively characterized human tumor-associated antigen) delivered intramuscularly in mice (Conry et al., 1995). Subsequently, the animals were subcutaneously injected with syngeneic, human CEA-transduced, colonic adenocarcinoma cells. This approach ellicited a high CEA-specific immune response and complete absence of tumors, probably due to either miocytes functioning as antigen presenting cells or providing an endogeneous source of CEA to draining lymph nodes.

MHC proteins have also been proposed as stimuli of antitumoral immunity. In this approach, recombinant allogeneic class I MHC DNA was retrovirus-transfected in mice bearing syngeneic experimental colon adeno-carcinomas or fibrosarcomas. Subsequently, a clinical protocol was devised for liposome-mediated transduction of the same gene within patients with metastatic melanoma at subcutaneous lesions. The objective was to induce an immune response to the foreign MHC antigen and possibly to other, previously unrecognized, tumor surface antigens in unmodified tumor cells (Nabel et al., 1993, 1994; Plautz et al., 1993; Culver et al., 1995). In mice, this technique induced attenuated tumor growth and several tumor regressions (Plautz et al., 1993). In one of the patients the injected lesion and distant metastases regressed; in the other four only the injected lesion responded (Nabel et al., 1993). Alternatively, transfected gamma interferon proved able to enhance MHC expression by tumor cells (Restifo et al., 1992).

For a given level of tumor cell antigen expression and MHC availability, the killing efficiency of TIL depends on their cytokine supply. The coercion could be alleviated if tumor cells or cytotoxic T cells themselves secreted the needed cytokines. This generated an approach termed ex vivo alteration of cancer cells (Schackert and Frost, 1993; Culver et al., 1995). It consists in obtaining a biopsy of tumor tissue which is digested with enzymes to obtain a cell suspension. Cells are grown in culture, transfected with the cytokine gene, irradiated to block their proliferative capacities and injected sub-cutaneously into the patient. A variant of this approach is to insert the cytokine gene into TIL. Gene-modified TIL were consistently found in the circulation of treated patients and they tend to selectively return in the tumor deposits though this latter property is still uncertain as revealed by studies on melanoma and renal carcinoma (Rosenberg et al., 1990; Merrouche et al., 1995). Still another variant, the use of transfected stromal fibroblasts as cytokine-producers gave unsatisfactory results (Schackert and Frost, 1993; Tsai et al., 1993).

The main cytokines in use, singly or combined, are interleukins (IL) 2 (Fearon et al., 1990; Gansbacher et al., 1990b; Gastl et al., 1992; Porgador et al., 1993; Schendel and Gansbacher, 1993), 4 (Tepper et al., 1989; Golumbek et al., 1991; Tepper, 1992; Cascinelli et al., 1994; Lotze et al., 1994), 6 (Porgador et al., 1992) and 12 (Caruso et al., 1996), gamma interferon ($IFN\gamma$) (Watanabe et al., 1989; Gansbacher et al., 1990a; Gastl et al., 1992; Restifo et al., 1992), granulocyte-macrophage colony-stimulating factor (GM-CSF, (Roth and Cristiano, 1997) and $TNF\alpha$ (Asher et al., 1991; Hwu et al., 1993). Due to its analogous mechanism of death induction we mention here Fas/Apo-1 gene transfer for human glioma cells (Weller et al., 1995). Animal and clinical studies have focused primarily upon melanoma, colorectal cancer, renal carcinoma and neural tumors (Culver et al., 1995; Roth and Cristiano, 1997). In animals immunized with the engineered cells tumors were suppressed or, at least, showed reduced growth and, generally, protection against subsequent challenge with the parental tumor cell lines (Fearon et al., 1990; Porgador et al., 1993; Culver et al., 1995).

All the above-mentioned immune-stimulating approaches share the final way consisting in the induction of active cell death. Indeed, transfected CEA or MHC produce CTL and NK accumulation in the neoplastic deposit (Culver et al., 1995), a process boosted by transduced cytokines. Particularly, IL 2 and 4 mediate the localization of killer cells to the site of the tumor and IL2 stimulates the proliferation and lytic capacities of NK cells and CTL, while inducing the differentiation of the former into LAK cells (Hancock and Rees, 1990; Gastl et al., 1992; Whartenby et al., 1995). The "lethal hit" induced by TIL is based on two

mechanisms probably coexisting within the same effector cell (Golstein et al., 1995; Nagata, 1997). The first is the production of Fas ligand (FasL) which stimulates Fas, a member of the TNF receptor (TNFR) family. Upon its activation, Fas recruits FADD (MORT1, Fas-associated protein with death domain) which itself binds and activates FLICE (FADD-like ICE, caspase 8) (Strasser et al., 1995). The second mechanism of TIL-induced apoptosis is the release of perforin and granzymes. It is thought that perforin produces pores in the membrane of the target cells through which granzymes are introduced to activate caspase 3 (cystein protease protein 32, CPP32) and thus start the caspase cascade. In this context, TNFα is particularly efficient since it does not only stimulate anti-tumor immunity but also directly induces apoptosis (Larrick and Wright, 1990) via TNFR1, another member of the TNFR family which functions in close analogy with Fas (Nagata, 1997) and through the increase of P53 levels (Gotlieb et al., 1994) being also able to induce necrosis (Laster et al., 1988). Finally, these ACD-inducing properties of TNFα are to be connected with the interactive tumor-cell killing between this cytokine and radiation (Sersa et al., 1988; Hallahan et al., 1989; Weichselbaum et al., 1994). It is also worth noting that interferons too, beside stimulating immunity, are direct inducers of apoptosis (Lokshin et al., 1995). Yet, mechanisms are complex enough to make predictable contradictory results. One important recent observation is that ACD is intrinsically immunosuppressive. Thus, the presence of apoptotic cells during monocyte activation increases their secretion of the anti-inflamatory IL 10 and decreases secretion of immunoinducers such as IL12 and TNFα (Voll et al., 1997). This may shed light upon the paradox of high ACD within tumors and set limitations for this promising type of therapy (Dickman, 1998).

d) Hormone ablation

While less important than other approaches, this type of therapy must be cited because it produced seminal investigations during the very discovery of apoptosis: castration (androgen fall) induces the regression of prostate cancer and so does estrogen ablation for breast tumors. This effect is mainly the result of ACD (Kyprianou et al., 1990, 1991; Westin et al., 1995).

e) Gene therapy

Gene therapy, the newest approach for cancer therapeutics, has focused on two goals, i.e, on the one hand using DNA constructs to potentiate existing therapy, such as radiotherapy, or to deliver more specifically existing therapy, such as chemotherapy via drug sensitivity genes or immunotherapy; on the other hand, DNA is transfected to revert the genetic profile of malignancy within tumor cells (Favrot et al., 1998). The broad spectrum of abnormalities make the results given by inactivation and replacement gene therapy unexpected since, at first glance, this approach ought to be able to correct all the genetic lesions in every tumor cell which is impossible both because not all the alterations are known and since vectors available have efficiencies far below 100%. Yet, it appeared that correction of a single essential gene abnormality within only a fraction of tumor cells can be sufficient to induce tumor repression, possibly because both copies of a tumor suppressor gene must be generally inactivated to eradicate its function (Weinberg, 1991). Therefore, replacement of only one normal copy of the gene in cells with homozygous loss of function can restore tumor suppression (Roth and Cristiano, 1997). In any event, the antitumoral effect relies strongly on the ability of transduced cells to induce apoptosis around them, in parental cells (Xu et al., 1997). This phenomenon was termed "by-stander killing"; it consists of the induction of apoptosis and was mostly investigated for suicide genes, in particular herpes simplex virus thymidine kinase (HSV-TK) (Samejima and Meruelo, 1995; Hamel et al., 1996). In vitro, the phenomenon relies on toxic-metabolite circulation between transfected and non transfected cells via apoptotic bodies (Freeman et al., 1993; Whartenby et al., 1995), gap junctions (Mesnil et al., 1996) or by diffusion in the culture medium (Chen and Waxman, 1995). These mechanisms have been proposed for p53-induced by-stander apoptosis of lung cancer cells in vitro (Cai et al., 1993; Negoescu et al., 1996a; Coll et al., 1998). In vivo, by-stander apoptosis may be favoured by increased cell-cell interaction (Wu et al., 1994) and by apoptotic-body phagocytosis, stronger in tissues than in culture (Arends et al., 1994; Negoescu et al., 1995). Furthermore, by-stander killing is boosted in vivo by an immune component which also allows the rejection of distant metastases and resistance to further rechallenges with non transfected tumor cells (Barba et al., 1994; Vile et al., 1994). Alternatively, conflicting results obtained with p53, a multifunctional gene acting far upstream in the ACD pathway, lead to target cancer gene therapy toward the final effectors of apoptosis (Favrot et al., 1998): $BclX_S$ (Clarke et al., 1995; Sumantran et al., 1995; Dole et al., 1996; Ealovega et al., 1996), caspases (Yu et al., 1996) and, most promisingly, Bax (Negoescu et al., 1996a; Sakakura et al., 1996; Strobel et al., 1996; Yin et al., 1997; Coll et al., 1998).

Interestingly, due to the central position of apoptosis in the action of almost all gene therapy protocols, more and more information is available about the involvement of transfection techniques in apoptosis initiation. And these may provide therapeutic tools.

Retroviruses, which integrate into genomic DNA, have been selected as the vectors in 80% of the clinical gene therapy trials approved. Yet, their long and delayed protein delivery may not always suit the need for acute treatment of tumors (Moolten, 1994). They are reputed harmless for their target cells (Vile and Russell, 1995).

The situation is different with adenoviruses (Pennisi, 1998). They are more and more used due to their higher

titers allowing greater transduction efficiencies, often around 100%. In tumors this is an advantage because the effect starts promptly; moreover, it is exerted for a brief interval since the gene resides in cells primarily as unintegrated, nonreplicating DNA susceptible to dilution as a result of cell multiplication (Moolten, 1994; Kroemer and Perricaudet., 1995; Zhang et al., 1995). Thus, DNA can be administred in a drug-like manner, repetitively and safely, on an "as-needed" basis. Adenoviruses produce several proteins which are implicated in apoptosis regulation in the host cell aiming at optimum virus replication. The main of these are the early (E) viral proteins E1A (which induces apoptosis by both p53-dependent and p53-independent mechanisms) and E4 (which initiates ACD independently of P53 expression) (Debbas and White, 1993; Lowe and Ruely, 1993; Teodoro et al., 1995). The viral e1B gene encodes two products that inhibit apoptosis, a 55 kDa protein which inactivates p53 and a 19 kDa protein which acts downstream, analogously to Bcl_2, to block Bax. Adenoviruses engineered for gene therapy have generally e1A and e1B deletions to preclude uncontrolled virus replication, but e4 has been mostly left functional and thus can exert its lethal effect (White et al., 1991; Rao et al., 1992; Boyd et al., 1994; Brough et al., 1996; Han et al., 1996). Data are lacking to evaluate the impact of adenovirus vector-induced or -repressed apoptosis during cancer gene therapy. However, at least one salient application of the above-described mechanisms has been tested under the form of an E1B-55kDa-deficient adenovirus, unable to bind and block p53 and thus replicating in and killing only p53-deficient tumor cells (Bischoff et al., 1996). In the same vein, recent data show the ability of reoviruses to replicate selectively within tumor cells with activated ras pathway, leading to tumor lysis (Coffey et al., 1998).

Cationic liposomes are an attractive method (termed lipofection (Felgner et al., 1997) for therapeutic DNA delivery to cancer cells. Their growing popularity is justified by relatively high transfection efficiency, low environmental toxicity and commercial availability (Farhood et al., 1995). Moreover, as they require no packaging cells, lipids are adapted to the transfection of highly cytotoxic DNA, such as ACD-inducing gene constructs. Cationic liposomes form complexes with DNA through charge interactions. The complexes bind to the negatively charged cell membrane due to the presence of excess positive charges in the complex (Gao and Huang, 1995). Then, complexes are taken up through spontaneous endocytosis resulting in endosomes filled with lipid-DNA particles. A small portion of the DNA is released into the cytosol from which it enters the nucleus for transcription. The majority of the DNA stays in the endocytic compartments where it is eventually degraded (Labat-Moleur et al., 1996). DNA liberation through endosome rupture is enhanced by the formation of lipid inverted hexagonal phase (HII)(Litzinger and Huang, 1992; Koltover et al., 1998) which is strongly connected to the presence of neutral components (such as L-dioleoyl phosphatidyl-ethanolamine, DOPE) in the lipid formulation (Farhood et al., 1995).

This complicated and still obscure sequence of phenomena may interact with cell viability, primarily concerning Bax function (Brady and Gil-Gomez, 1998). Indeed, recent results link the apoptotic effect of Bax to its moving from the cytosol to mitochondria (Hsu et al., 1997; Wolter et al., 1997; Zhang et al., 1998). Localization of Bax to mitochondria may trigger a permeability transition by forming channels in lipid membranes, critical events during early stages of ACD blocked by Bcl_2 (Antonsson et al., 1997; Schlesinger et al., 1997; Ishibashi et al., 1998). We are currently investigating Bax delocalization using a model of tissular ACD chronology, the Graves-Basedow thyroid (Labat-Moleur et al., 1998, 1999). The Golgi cisternae would be the starting point of Bax activation through the cytosol to mitochondria. Thus, during apoptosis initiation, there occurs a change in Bax immunolabeling pattern from a small apical paranuclear dot, probably within the Golgi apparatus, in non apoptotic thyrocytes to a dominantly diffuse microgranular distribution, similar to that of Bcl_2. This is suggestive for a delocalization of Bax from a storage compartment to its accepted site of action, mitochondria (Mignotte and Vayssiere, 1998) where it likely opposes the ACD inhibiting function of Bcl_2, weakly expressed at this moment while it is strong when Bax is collected in the paranuclear dot. Interestingly, groups of cells with no other morphological indications of ACD showed contrasted and concordant Bax-Bcl_2 patterns: Bax dot and strong Bcl_2 on the one hand, versus dominantly microgranular Bax and weak Bcl_2 on the other hand (Labat-Moleur et al., 1999).

The large-scale interference between lipofection and internal cell membranes (Labat-Moleur et al., 1996) can thus be considered as able to favor Bax delocalization and ACD induction. Indeed, the paranuclear Bax dot in non apoptotic thyrocytes was also found in a lymphoma (Schlaifer et al., 1996), and recently published data indicate that lipofection induces apoptosis in lymphocytes (Ebert et al., 1997).

Hypotheses

Current evolutions and promising areas for proximal future investigations.

a) Cancer gene therapy based on final inductors of apoptosis (Figure 1)

The above data provides the general feature of gene therapy of malignant disease as an attempt to induce apoptosis. And since both gene therapy and apoptosis are recent fields of research, with virtually all their history spanning over the last two decades, they tend to merge. The result of this tendency is targeting cancer gene therapy toward the progressively discovered final steps of apoptosis induction, primarily Bax (Negoescu et

al., 1996a; Sakakura et al., 1996; Strobel et al., 1996; Yin et al., 1997; Coll et al., 1998) and caspases (Yu et al., 1996). This trend is based upon the assumption that treatment efficiency is inversely proportional to the length of the downstream pathway between the function of the transfected gene and cell execution. Conflicting results obtained with p53, a gene acting far upstream in ACD induction, are a good example favouring the current tendency (Favrot et al., 1998). So, many results to come in cancer gene therapy may issue from progressive understanding and manipulation of the final, immediately acting, segment of the apoptotic executioner, best suited to bypass tumor cell resistance to ACD.

b) Endogeneous caspase inhibitors: a general protection against apoptosis

Despite the high level of apoptosis in tumors the complete resistance to ACD induction of some malignant clones is the basis of cancer therapy failure. Hypotheses to explain this limitation exist concerning every effector of the apoptotic pathway and we have evoked the main ones of them. However, a promising attempt for a general explanation may be currently on the way: caspase inhibition.

Indeed, while various inhibitors are widely used to investigate caspase function (Livingston, 1997), little attention has been paid to caspase blockers in tumorigenesis. As results begin to collect, it turns out that experimental inhibition of proteolysis eliminates ACD induced by many (virtually all) mechanisms and does provide protection to malignant cells (Bruno et al., 1992; Dou et al., 1997; Lynch et al., 1997). The most recently suggested mechanism would be that caspase inhibitors hinder the targeting of Bax to mitochondria (Goping et al., 1998). Moreover, the inhibition of caspase activity induces a switch from apoptosis to necrosis, which suggests that both share common initiation pathways, the final issue being determined by the presence of an active caspase (Lemaire et al., 1998). This may be in connection with the bad prognosis of necrosis-rich tumors, since these are known to contain little ACD (Wyllie, 1985; Sarraf and Bowen, 1988; Bursch et al., 1990; Arends et al., 1994; Staunton and Gaffney, 1995; Collins et al., 1997; Messam and Pittman, 1998; Soini et al., 1998).

The above data strongly suggest the existence of natural (endogeneous) protease inhibitors at work within cancer cells. Indeed, cells are able to produce inhibitors of proteolysis. The main one of these is α_2-macroglobulin (α_2M). α_2M is a large (720 kDa) plasma glycoprotein that inhibits virtually all proteases (Barrett, 1981; van Leuven, 1984). Although circulating α_2M originates from hepatocytes, numerous cell lineages are known to synthesize it (Feige et al., 1998). A possible implication of α_2M in apoptosis has been first suggested for adrenocytes. Thus, a significant part of the bovine adrenocortical cell population originating from the fasciculata-reticularis zone undergoes apoptosis when grown in primary culture in the absence of adrenocorticotrophic hormone (ACTH) (Negoescu et al., 1995). In these conditions, the surviving cells synthesize huge amounts of α_2M (Negoescu et al., 1994; Savona et al., 1994). Moreover caspases act on nuclear proteins of malignant cells (Nakagawara et al., 1997), presumably during the early apoptotic stage termed "swelling cells" (Desjardins and MacManus, 1995). At this step, chromatin begins its condensation at the margin of the nucleus and starts breaking (Labat-Moleur et al., 1998, 1999). Precisely, mildly broken DNA forms in vitro complexes with α_2M (Cheng et al., 1983). Finally, strong synthesis of α_2M was noted in therapy-resistant H-322 and H-358 bronchioloalveolar carcinoma cell lines (Negoescu et al., 1996a; Coll et al., 1998; and Negoescu, unpublished results).

Conclusion: By-stander apoptosis (the advantage of in vivo cancer therapy)

Taken together, the above data point at the counterintuitive fact that the attempt to induce apotosis is favoured by a tumoral environment. While mechanisms underlying this peculiarity are far from being elucidated, the trend is not totally unexpected. One could argue that, considering CLF values, most cancers meet apoptosis-inducing agents with a sort of "spontaneous by-stander killing" exceeding 90% of the newly produced tumor cells. Thus, the titles of two seminal papers may serve to formulate a conclusion for this review. W.T. Shier asked (Shier, 1988): "Why study the mechanisms of cell death?". The answer had been given by Kerr and Searle (1972): because it can provide "A suggested explanation for the paradoxically slow growth rate of (...) carcinomas that contain numerous mitotic figures": most of the cancer cells naturally die. It also probably provides the main (or only) way to therapy: inducing the remaining 10% of truly immortal cells to apoptosis.

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