Journal of BUON **10:** 309-318, 2005

CONTINUING EDUCATION IN ONCOLOGY

Molecular mechanisms of cancer cells survival

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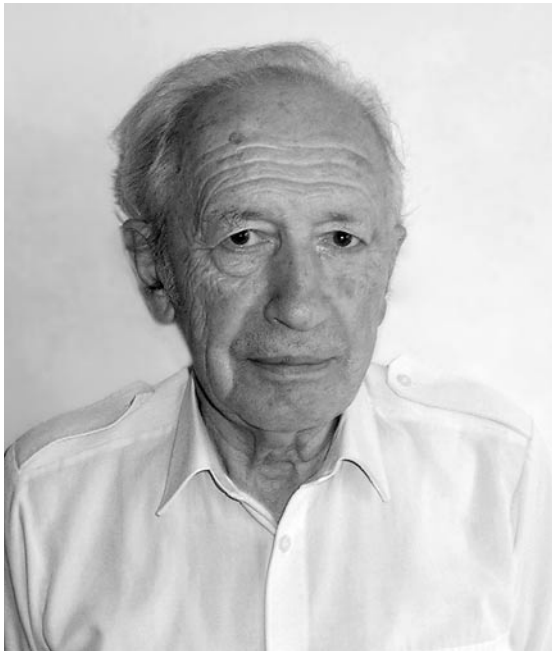

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Summary

It is of great interest for cancer therapy to elucidate and overcome the mechanisms that cancer cells develop to fight back the defense systems of the organism and anticancer treatments. The anticancer defense of the organism consists of four processes: 1. Activity of the cellular immunity; 2. Production of cytokines; 3. Activation of tumor-suppressor genes; 4. Blocking of the enzyme telomerase.

Several molecular mechanisms that cancer cells develop for survival are described: 1. Reactivation of the telomerase; 2. Suppression of apoptosis; 3. Elimination of effector cells; 4. Shedding of soluble receptors; 5. Neutralization of tumor-suppressor genes; 6. Developing of a drug efflux pump; 7. Neoangiogenesis; 8. Overcoming and utilizing hypoxia; 9. Other rescue mutations; 10 Access-restriction factors.

Some new approaches for overcoming the survival mechanisms of cancer cells are briefly outlined.

Key words: anticancer defense, cancer cell survival, cellular immunity, genes, molecular mechanisms, telomerase

Introduction

Understanding the mechanisms that allow cancer cells to defeat the natural defenses of the organism and to survive anticancer treatments is of crucial importance for the development of a successful therapy. These mechanisms can only be uncovered at the molecular level. We shall discuss the problem of how cancer cells survive in the light of important data obtained in recent years and from the point of view of the cellular regulatory genetic networks controlling the metabolism and all cellular functions.

Genes and genetic networks

To understand how the malignant phenotype arises we have to answer the question whether the changes involved are a problem of genes only, or essentially, of gene regulation. Many data show that genes alone cannot determine the phenotype: 1) It has been firmly established that heterologous gene transfer cannot change the phenotypic features of the host. Human genes, for instance, have been transferred to mice, to yeast and to bacterial cells, where they remain fully active without affecting the morphological features of these organisms; 2) Genes controlling basic cellular functions such as cell cycle, embryonic development, transcription, housekeeping genes, histone genes, display a high degree of evolutionary conservation [1]; 3) In terms of informational content, humans and chimpanzees for example are 99%

identical. It is clear that additional information is needed to translate a set of genes into a given phenotype.

Several authors have already suggested that the important factor is the "architecture" of the regulatory system [2-4]. It is essential to point out the integrating function of the genome: genes do not function as separate units but are interconnected in regulatory circuits – *genetic networks* –, which have been formed in evolution to control the metabolic program of the cell, and all its other functions [1,5]. These networks are based on specific regulatory genes which code for proteins (trans factors) that bind to specific DNA sequences (cis elements), such as promoters, silencers, enhancers, upstream activating sequences [6].

The genetic network of a given species has been established in the evolution as a functionally stable structure. The intergenic interactions depend on many parameters which determine the phenotype and the functional state of the cell-such as the equilibrium constants of reversible interactions between regulatory proteins and DNA, the permeability of cellular membranes, cellular configurations, etc. All of these parameters ultimately depend on DNA sequences. Mathematical analysis of these DNA/protein interactions - based on the law of mass action - shows that the genome is a nonlinear system. This means that changing its parameters within certain limits does not cause any significant perturbations. However, even very small alterations of the parameters outside these limits may drastically alter the properties of the system [1,5,7]. For a cell this would lead to only three possible outcomes: cellular death, arrest of cellular proliferation (lucky events) or the emergence of a new, malignant phenotype.

Anticancer defense systems of the organism

Under the effect of different factors, DNA often acquires mutations that affect the genetic network. In most cases this leads to cellular death but some give rise to malignantly transformed cells. The organism has developed defense systems to eliminate such cells. These systems include mainly four processes: 1. Cellular immunity; 2. Production of cytokines; 3. Activation of tumor suppressor genes; 4. Blocking the enzyme telomerase.

1. *Activity of the immune system - cellular immunity*

This defense is mounted by special effector cells which kill, lyse and phagocytose foreign pathogens and malignantly transformed cells. The effector cells are represented by several species:

a) Natural killer cells (NK); b) Lymphokine-activated killer cells (LAK); c) Macrophages; d) Antigen-presenting cells (AP-cells, also called dendritic cells-DC); e) CD4+ T-helper lymphocytes; f) CD8+ cytotoxic T-lymphocytes; g) Tumor-infiltrating leukocytes (TIL); h) Polymorphonuclear leukocytes; i) Antibody-dependent cytotoxic cells (ADCC); j) Thrombocytes which can also be stimulated to cytotoxic activity [8].

Two types of T-lymphocytes, Th1 and Th2, play an important role in the immune activity. They exert mutual repression upon each other: Th1 cells produce cytokines that stimulate cellular immunity and repress Th2 cells, while the latter activate humoral immunity and repress Th1 cells (Slide 1). Depending on many external and internal factors the CD4+ lymphocytes respond to antigens by developing Th1 or Th2 phenotype. This phenotype depends also on the balance between interleukin (IL)-12 and IL-4, the former stimulating a Th1, while the latter a Th2 response. Zn deficiency [9] and poisoning with Pb [10] shift the response from Th1 to Th2. Prostaglandin E2 also decreases Th1 activity [11].

It should be stressed that Th1 cells possess several activities directed towards eliminating tumor cells: 1. Antiproliferative; 2. Tumorocidal; 3. Activation of cellular immunity; and 4. Modulation of gene expression. All of them are critical for the anticancer defense, which is therefore favored by all factors inducing a Th2 to Th1 shift.

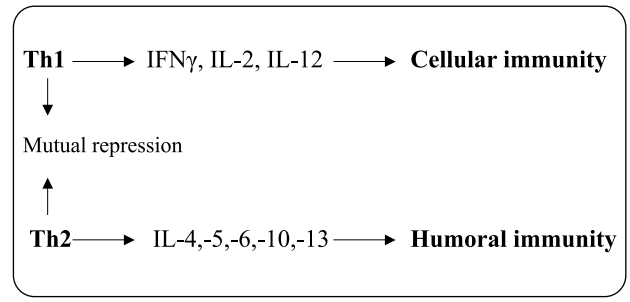
1.1. The *antiproliferative* activity is due to:

a) Inhibition of oncogene expression, e.g. of c-Myc, of c-erbB-2 etc. [12, 13].

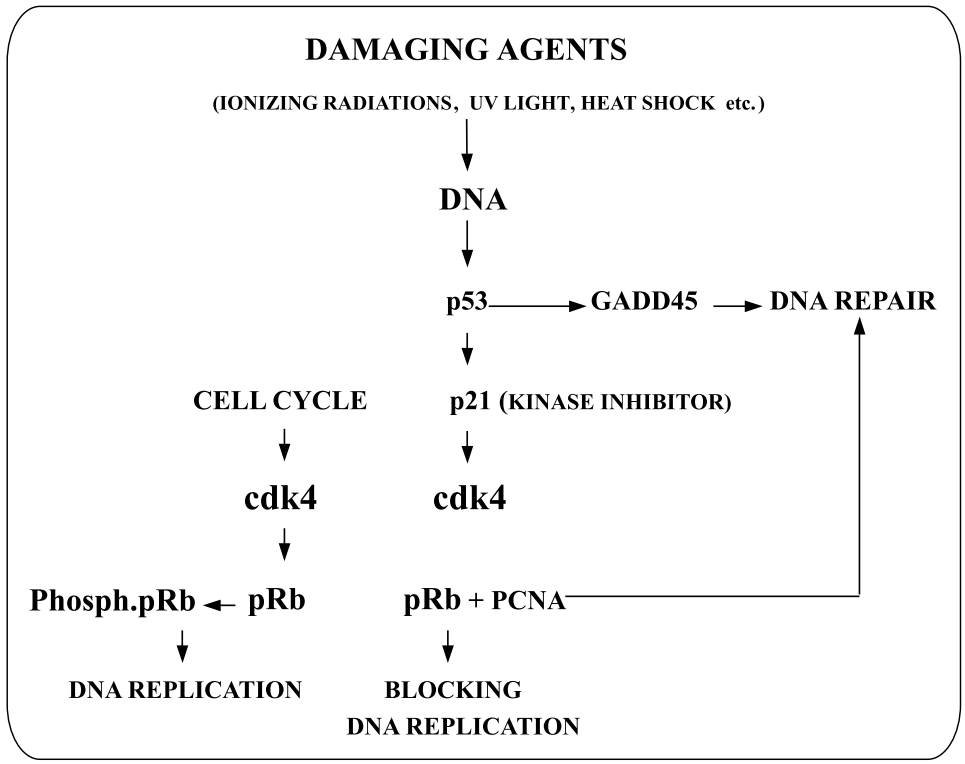
b) Induction by interferon (IFN) γ of the enzyme indolamine-2-3-dioxygenase (IDO) [14], that destroys tryptophan, thus leading to tryptophan starvation.

c) Inhibition of transferring receptors expression [15] causing iron deficiency.

d) Induction of the kinase inhibitor p27, thus blocking the cell cycle at the end of G1 [16, 17] (Slide 2).



Slide 1. Relationship between the two types of thymocytes, Th1 and Th2, responsible for cellular and humoral immunity, respectively.



Slide 2. Schematic representation of the processes leading to a normal cell cycle or to blocking DNA replication.

e) Inhibiting the synthesis of growth factors (for instance IL-2, which is the growth factor of multiple myeloma) [18].

f) Stimulation of fibronectin synthesis [19], which is inversely correlated with cell proliferation.

g) Inhibition of DNA polymerase [20].

h) Induction of calcium/calmodium-dependent enzymes and death-associated proteins (DAP), leading to apoptosis [21].

1.2. The *tumorocidal* activity is dependent on the antiproliferative activity on one hand, and on the other on the cytotoxicity of effector cells.

1.3. The *immunomodulatory* activity consists of:

a) Increasing the synthesis of class I and inducing the synthesis of class II antigens of the major histocompatibility complex (MHC I: HLA-A,B,C and MHC II: HLA-DR, DQ, DP).

b) Stimulation of Th1 and suppression of Th2 proliferation.

c) Activation of co-stimulatory molecules in the cell membrane – ICAM-1 (CD54), LFA-3 (CD58), B7-1 (CD80), B7-2 (CD86), which ensure the adhesion of effector cells to the target cells in order to accomplish the cytotoxic effect.

d) Activation of some components of the complement (C2, C4, factor B) [22], that increase the antibody-dependent cytotoxicity of macrophages.

2. ***Production of cytokines***

These are proteins that fulfill intercellular communications in the same tissue or among different tissues. Although structurally different, they all affect cellular activity. At present the following groups are known:

a) Interferons (IFN-α, IFN-β, IFN-γ, IFN-ω).

b) Interleukins (IL-1 to IL-18).

c) Colony-stimulating factors: granulocyte (G-CSF), macrophage (M-CSF) and granulocyte-macrophage (GM-CSF).

d) Growth factors.

e) Transforming growth factors (TGF-α and TGF-β).

f) Fibroblast growth factors - acid and basic (aFGF and bFGF).

g) Tumor necrosis factors (TNF-α and TNF-β).

h) Chemokines – a family of proteins playing a key-role in T-helper cells migration.

3. ***Activation of tumor suppressor genes***

During the recent 15 years several genes have been discovered that can suppress carcinogenesis. One of the most important tumor suppressor genes is the gene coding for the protein p53. For many years it was considered to be an oncogene since more than

50% of human tumors were found to be associated with mutations in this gene. However, p53 was shown subsequently to be involved in protecting the genome from inheriting damaged DNA and was called "guardian of the genome" [23,24]. This gene is induced by many factors that damage DNA (Slide 2). The cell cycle is normally accomplished with the help of a cyclin-dependent kinase (cdk) which hyperphosphorylates the retinoblastoma protein pRb and thus removes its inhibitory effect on DNA replication. When DNA is damaged, production of p53 induces the synthesis of the kinase inhibitor p21, the phosphorylation of pRb is prevented and DNA replication is blocked. The molecular machinery of the cell is directed towards DNA repair (Slide 2). By arresting the cell cycle until DNA is repaired, and in some cases inducing apoptosis, p53 fulfills its most important function – to prevent the damaged DNA from being inherited by daughter cells, which opens the possibility of malignant transformation.

4. *Blocking the enzyme telomerase* [25,26]

The ends of chromosomes contain hundreds of copies of repeated nucleotide sequences called telomeres whose main function is to prevent chromosome fusion and to protect the chromosomes from exonucleolytic attack. Telomeres cannot be fully replicated by the same enzymes that replicate DNA. To achieve this process a special enzyme is employed, called telomerase – a RNA-protein complex functioning as a reverse transcriptase. Telomerase RNA is complementary to the 3'single-stranded overhanging G-rich telomeric strand at the end of the chromosome. During DNA replication the telomerase binds to this end and a complementary telomeric sequence is synthesized. The second strand is synthesized by a cell polymerase. As a result the telomeric DNA is complementary to the RNA component of the telomerase.

Telomerase is normally active during embryonic development, while in the adult organism it is inactivated. Therefore, during each cell division the chromosomal ends are shortened until important genes are affected and the cell dies. In this way a normal adult cell is programmed to undergo a limited number of cell divisions only.

Mechanisms of cancer cells overcoming the anticancer defense. The role of rescue-mutations

As discussed above, malignant cells have a destabilized genetic network leading to a disturbed regulatory system and altered gene interrelations, with some active genes repressed and some silent genes activated. Due to the emergence of an unstable genetic network further changes take place manifested as *tumor progression*. The various mutations that occur affect different cellular targets including membrane receptors, metabolic pathways and the nucleus. They make the cellular population very heterogeneous and subject to selection pressure under the effect of internal and external factors including chemotherapeutics. In this way some malignant cells survive and emerge as resistant to anticancer therapy. The following self-rescuing properties of malignant cells are well known:

1. *Reactivation of telomerase*

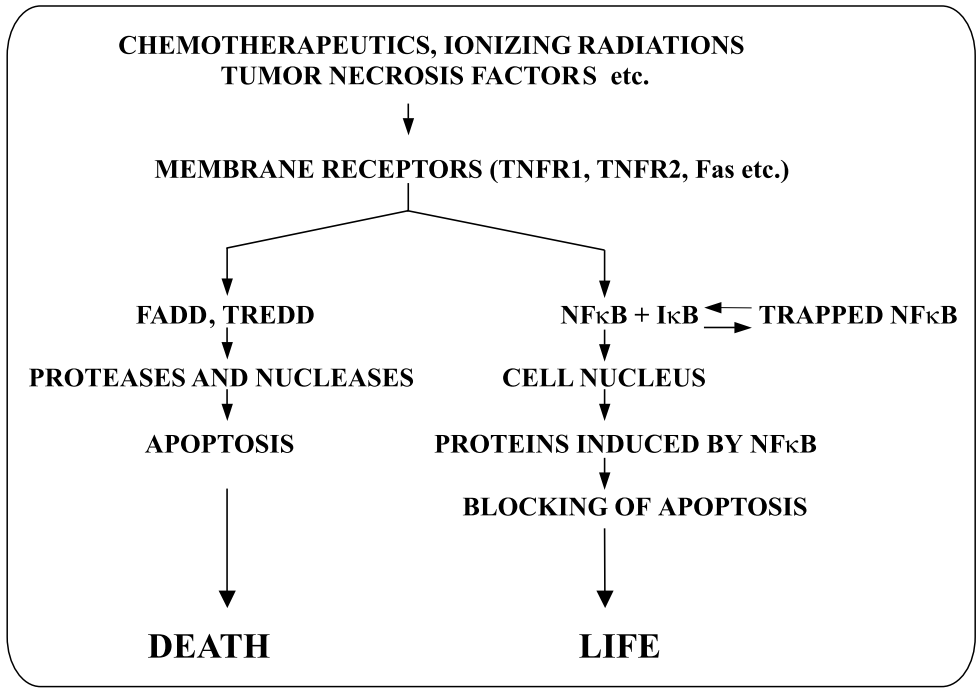
In cancer cells telomerase is reactivated ensuring unlimited number of cell divisions [27-29]. The mechanism of this reactivation is not known, neither is the mechanism of its inhibition. It seems that telomerase-independent mechanisms also exist since in some human cell lines telomere maintenance is observed in the absence of telomerase activity [25].

2. *Suppression of apoptosis*

Apoptosis is sort of programmed cell death. Preventing apoptosis is a powerful mechanism opening the way to carcinogenesis. There is an essential difference between the passive process of necrosis and that of apoptosis [30-32]. The latter is an active process starting with the synthesis of cell-cycle specific proteins, but ending with cellular death and showing specific morphological features. It is considered as an abortive cell cycle [33-38].

Apoptosis is particularly important for cancer therapy [39,40]. Anticancer factors kill cancer cells by apoptosis. This process is initiated by the interaction between specific cellular receptors and their ligands (e.g. FasL] or under the effect of various physical and chemical agents (ionizing radiations, antimetabolites etc.). At the same time there is a family of transcription factors in the cytoplasm, known as nuclear factors kappa B (NFκB), which enter the nucleus and induce proteins inhibiting the apoptotic process [41-43]. Also found in the cytoplasm is an inhibitor IκB [44-46] which binds and partially traps NFκB, as shown in Slide 3. As a result, the cell is at the boundary between life and death [47]. Depending on the strength of interaction between NFκB and IκB, the cell may survive or may enter the apoptotic pathway.

Thus NFκB is a survival factor for cancer cells and, therefore, a target for cancer therapy. Breast cancer



Slide 3. Induction and blocking of apoptosis.

cells are a good example of survival due to constitutive expression of NFκB, which prevents their apoptotic death, unlike normal mammary epithelial cells which, under the same conditions, die of apoptosis [48,49]. During tumor progression the sensitivity of cancer cells to the apoptotic ligand decreases [50].

3. *Elimination of effector cells*

This defeats cellular immunity and is achieved by cancer cells in several ways:

a) By the expression of ligands which induce apoptosis in effector cells. For example, the expression of FasL on the membrane of tumor cells. This ligand binds to the Fas receptor, normally expressed on the surface of TILs, and induces their apoptotic death [51,52].

b) By secreting factors suppressing the locomotion of effector cells and their penetration through the extracellular matrix, preventing their migration towards the target cells [53].

c) By expression proteins that suppress the lysis of cancer cells by LAK [54].

4. *Shedding of soluble receptors*

In some cancer cells proteases are expressed which cleave off the extracellular domain of receptors or other surface antigens (e.g. sIFNγR, sICAM-1, sFas etc.), releasing them into the surrounding medium where they are able to bind the corresponding cytokines or ligands, thus blocking the immune reaction or apoptosis [55-57].

5. *Neutralization of tumor suppressor genes*

Many virus-induced tumors owe their emergence and survival to products that bind and neutralize p53. Such are the large T antigen of SV-40, the 55kD protein of the E1B adenoviral gene, the IE84 protein of CMV, the protein X of hepatitis B virus, and also the products of some eukaryotic genes such as the heat shock protein 70 and the MDM2 gene [24].

6. *Development of a detoxicating efflux pump* [58]

Some tumors show an initial response to therapy, but nevertheless subsequent relapses occur frequently. The recurrent tumors and their metastases are refractory to further treatment even to protocols involving multiple drugs aimed at different cellular targets. This multidrug resistance (MDR) is a widely studied cellular transport-mediated resistance. The classical MDR is characterized by:

a) Cross-resistance to chemically-unrelated drugs.
b) Decreased intracellular drug accumulation.
c) Overproduction of plasma membrane glycoproteins due to overexpression of the *mdr* gene.
d) Reversal to drug sensitivity by MDR modulators.

The most widely studied glycoproteins involved in MDR are the P-glycoprotein (Pgp), the MDR resistance-associated protein (MRP1) and the lung-resistance-related protein (LRP).

All these glycoproteins are localized in the cell membrane and contain binding sites for the toxic agents. Using the energy of ATP they expel the toxic substances out of the cell. MDR modulators block the drug efflux by a competitive or non-competitive way, i.e. by binding the modulators either to the same drug-binding sites, or to other sites causing alosteric changes inhibiting drug binding.

7. *Neoangiogenesis*

The formation of new blood vessels is necessary for tumor growth and for the permanent tissue reorganization that takes place in the tumor. This depends on the release of the vascular endothelium growth factor (VEGF) [59] which ensures the tumor survival. Some authors identify the enzyme thymidine phosphorylase with VEGF [60]. The life or death of the tumor cells is determined by the balance between the angiogenic factors and the anti-angiogenic chemokine IP-10 [61, 62].

8. *Overcoming and utilizing hypoxia*

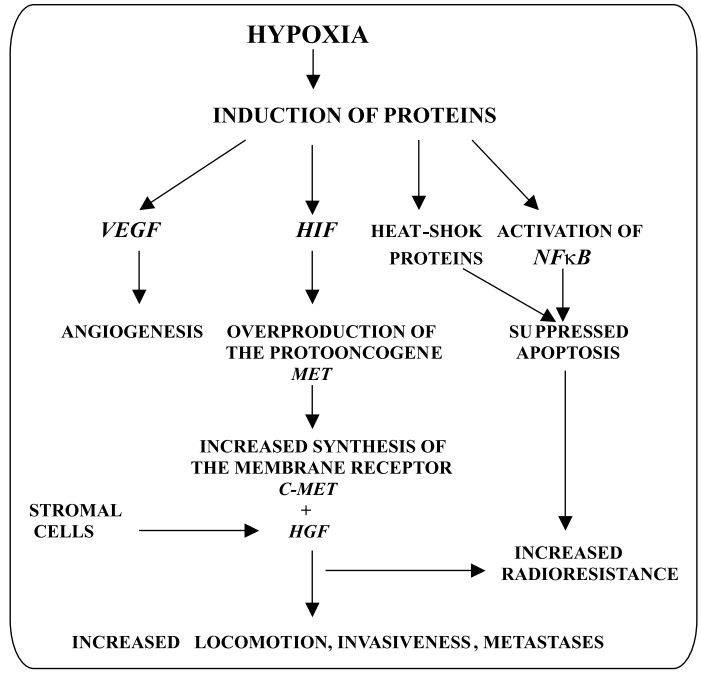
In 1970 Folkman put forward the idea of eliminating cancer cells by inhibiting angiogenesis, thus causing hypoxia [63]. Clinical data, however, did not confirm this prediction. Recently it was elucidated how cancer cells counteract hypoxia [64-68]. It was found that hypoxia induced proteins - hypoxia-induced factors (HIF) – that led to induction of VEGF and activated the transcription of the protooncogene *met* which increased the synthesis of the membrane receptor c-Met, a tyrosine kinase. The latter binds the hepatocyte growth factor (HGF) produced by the normal cells of the neighboring stroma and enhances the invasiveness and metastatic properties of the malignant cells (Slide 4). HGF also stimulates neoangiogenesis, an effect mediated by a platelet-activating factor (PAF) synthesized by macrophages [69].

Hypoxia also induces heat-shock proteins, suppresses apoptosis by activating NFκB and increases the radio resistance of malignant cells. It makes these cells resistant to the antiproliferative activity of interferons [70].

As a result, instead of killing the malignant cells, hypoxia makes them more aggressive, more metastatic and more resistant to anticancer treatments. This weakens the hope of eradicating tumor cells by anti-angiogenesis, although such an approach is under clinical trial.

9. *Other rescue mutations*

a) In hematological malignancies great quantitative variations are observed in the expression of IFNγ receptors. Of 77 different malignant types studied, 6 lymphoid leukemia types did not express these receptors that made them insensitive to this cytokine [71].



Slide 4. Effects of hypoxia on cancer cells.

b) The metabolic chain inducing IDO by IFNγ is interrupted in some cancer cells, tryptophan can not be degraded, and the cells survive [72].

c) A metabolic pathway is interrupted by mutations in the tyrosine kinase JAK-1, that makes the malignant cells insensitive to all three types of IFN [73].

10. *Access-restriction factors*

These are either natural barriers or restrictions induced by cancer cells.

a) A typical case of natural barrier is the blood/lung barrier – the inability of parenterally applied immune interferon to penetrate lung alveoli [74-76].

b) Most of the cells within a large tumor mass are also inaccessible to the cellular immune system.

c) Inhibited expression of ICAM-1 prevents the contact between cytotoxic leukocytes and target malignant cells.

d) Inhibiting TILs locomotion and their penetration through the extracellular matrix also prevents their contact with malignant cells [53].

e) Disturbance in the signaling pathways of kinases KKI and CK2 increases their level, leading to elevated NFκB seen in primary human mammary tumors [77].

f) Mutations in the kinase inhibitor p27 disturb its relations with cdk2 and eliminate the block of the entry into S phase [16, 17].

g) Mutations in the metabolic pathway of IL-2 prevent the induction of LAK activity [78].

Overcoming the survival mechanisms of cancer cells

For cancer therapy it is important on one hand to suppress the mechanisms rescuing cancer cells, and on the other to make cellular immunity more effective in killing the heterogeneous malignant cell population.

1. *Suppressing mechanisms*

a) Inhibition of the survival factor NFκB would be a potential way for improving cancer therapy [79]. A mutant of IκB (a super-repressor) has been found, which strongly binds NFκB and irreversibly leads to apoptosis [46].

b) In various tumors apoptosis was induced by vector-dependent over-expression of the CID gene that codes for a DNA-binding protein activating p53 [80].

c) The detoxicating function of cancer cells can be eliminated by a number of MDR modulators [58].

d) The receptor profile of malignant cells can be also modified. Doxorubicin for example enhances the expression of both Fas and FasL so that the malignant cells mutually kill themselves by apoptosis [52]. Methotrexate also induces the expression of FasL in the membrane of leukemia cells and, due to the presence of Fas, the malignant cells die of apoptosis [81].

e) It would also be rational to use factors that shift the thymocyte profile from Th2 to Th1 phenotype in order to strengthen the immune reaction.

f) An approach to the problem of hypoxia would be to combine anti-angiogenesis with suppression of genes responsible for the synthesis of VEGF and proteins such as Met.

2. *Effective cellular immunity. Anticancer vaccines*

The most difficult problem in cancer therapy arises from the numerous mutations creating a heterogeneous cell population of malignant cells. In order to obtain effector cells able to attack all these different cells the attention recently has been focused on elaborating anticancer vaccines [82, 83]. To this end antigen-presenting (AP) cells (called dendritic cells, DC) are loaded *ex vivo* with all different tumor antigens which they present to the effector cells, thus creating tumor-specific toxic T-lymphocytes. The latter are introduced into the lymphoid organs of the individual of their source. Several antigen-loading procedures have been developed. An excellent source of all various tumor antigens are apoptotic cancer cells [84-86]. This approach has proved to be efficient in mice [87] and such vaccines are already under clinical trials [88].

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