Biochemistry (Moscow), Vol. 68, No. 9, 2003, pp. 958-962. Translated from Biokhimiya, Vol. 68, No. 9, 2003, pp. 1173-1177.

MINI-REVIEW

Microbial Therapy of Cancer: Induction of Apoptosis, Recombinant Vaccines, and Inhibition of Angiogenesis

V. D. Samuilov

Department of Physiology of Microorganisms, Faculty of Biology, Lomonosov Moscow State University, Moscow 119992, Russia; fax: (7-095) 939-3807; E-mail: vds@8.cellimm.bio.msu.ru

Received January 30, 2003
Revision received March 3, 2003

Abstract—This review presents diverse approaches to cancer biotherapy including bacteriolytic therapy aimed at inducing apoptosis in tumor cells, bacterial vector-based cancer vaccines, and inhibition of tumor neoangiogenesis by microorganisms. The normal microflora in humans and animals may restrain malignant tumor regeneration.

Key words: cancer, bacteriolytic therapy, vaccines, angiogenesis

In view of the inexhaustible metabolic potentialities of microbes and their considerable adaptability, one can rightfully make the statement that "Microbes can do everything". This statement, though, is usually interpreted only in the sense that microorganisms are involved in substance turnover in nature, that they interact with abiotic factors. However, of paramount importance (in the biological viewpoint) is the role of microorganisms in intercellular and interorganismic interactions. Relevant topics of interest include intercellular communication in microorganisms, the interactions between microorganisms and animals/plants, the driving forces of these interactions, and the regulatory mechanisms involved.

As far as the interactions of microorganisms with animals and humans are concerned, microbial agents exert a considerable influence on the physiological state of the macroorganisms. This can be nicely exemplified by the indisposition caused by intensive antibiotic therapy that inhibits normal intestinal microflora, disturbs the biological equilibrium, and thereby provides for the development of opportunistic pathogens. Do malignant tumors result from a disturbance of the biological equilibrium? Making good use of microorganisms, we can succeed in suppressing tumor growth, reverse their development, and, in some cases, cure the organism. Taking account of the new options offered to us by the biotherapy of cancer-related diseases, we give an affirmative answer to the above question.

BACTERIOLYTIC THERAPY OF CANCER

Current strategies of cancer therapy are based on radiation treatment and the use of drugs. Neither approach is sufficiently effective [1]. Most malignant tumors contain large, poorly vascularized areas (the formation of new blood vessels supplying a tumor with nutrients lags behind the growth of the tumor) characterized by hypoxia or anoxia. These zones are less sensitive to ionizing radiation, because radiation effects require oxygen. The delivery of chemotherapeutic agents is also difficult, because they are transferred via the bloodstream.

Schematically, the spatial structure of a tumor is spherical. The oxygen-rich outermost region of the sphere contains proliferating cells; the oxygen-depleted middle region consists of dormant cells, and the innermost region of the tumor is the anaerobic necrotic zone [1]. Hence, some parts of a tumor provide optimum conditions for the development of anaerobic bacteria.

A number of facultative and obligatory anaerobes that were introduced into solid tumors *in vivo* took residence in their hypoxic areas and propagated in them, resulting in the death of the tumor cells. A variety of test models were used, including sarcoma, rhabdomyosarcoma, carcinomas, melanomas, and breast cancer. The following bacteria were tested: *Clostridium* (the obligatorily anaerobic representatives *C. histolyticum*, *tetani*, *butyricum*, *acetobutylicum*, *pectinovorum*, *tyrobutyricum*,

0006-2979/03/6809-0958$25.00 ©2003 MAIK "Nauka/Interperiodica"

beijerinckii, and *novyi*), *Bifidobacterium* (the obligatorily anaerobic species *B. bifidum*, *infantis*, and *longum*), *Salmonella typhimurium* (a facultative anaerobe), and *Corynebacterium parvum* (an obligatory anaerobe) [1]. Among the anaerobic bacteria (including human pathogens such as *Cl. histolyticum* and *Cl. novyi* that cause gas gangrene and *S. typhimurium*, a salmonellosis pathogen) listed above, *B. bifidum* should receive special attention. This actinomycete, a representative of the useful microflora of the human intestines, is used for preparing (i) the bacterial medicines bifidobacterin or bificol (if combined with a medicinal strain of *Escherichia coli*) and (ii) fermented milk products (bifidoc). Anaerobic bacteria have been employed for treating cancer for a long time—for almost 150 years as noted in [1].

In experimental studies, some test animals (most studies used mice) with malignant tumors completely recovered, and regression or localization of tumors and retardation of their growth occurred in other animals. The test animals were infected by intratumoral or intravenous injection of bacteria or their spores [2]. Among the 26 tested strains of various bacteria, *Cl. novyi* proved to be most efficient; it can be rendered unable to produce its lethal toxin by deleting the relevant gene [2]. The use of this nontoxic strain of the anaerobe *Cl. novyi* in combination with conventional chemotherapeutic agents that kill the cells of the vascularized tumor areas is referred to as combination bacteriolytic therapy (COBALT). COBALT caused a rapid regression (within 24 h) of tumors in mice that were implanted with colon cancer HCT116 cells [2]. Using intravenous injections to deliver bacteria to tumors *via* the bloodstream is also a promising method of bacterial therapy of diffuse tumors that are dispersed in the patient's body. The combined method also proved efficient in *in vitro* studies with HCT116 tumor and B16 melanoma cells.

The point to be clarified concerns the mechanism of the destructive effect of bacteria on tumor cells. Does the bacteriolytic effect observed in tumor cells possess specific properties that differ from those of a similar effect occurring in normal (non-tumor) cells? What is the mechanism of cell death?

The physiological mechanism involved is based on programmed cell death referred to as apoptosis (reviewed in [3]).

As noted in [4], introducing toxic factors into the cytoplasm of a phagocyte cell is a typical strategy of pathogens that results in the death of the phagocyte. For instance, *Shigella* and *Salmonella* representatives penetrate into intestinal epithelial cells using the endocytosis mechanism. This causes acute inflammatory symptoms. *In vitro* studies revealed that these bacteria induce macrophage apoptosis using proteins IpaB [5] and SipB [6] secreted by them. In the macrophage cytoplasm, these proteins bind to caspase-1, but not to caspase-2 or caspase-3. The macrophage's death does not depend on caspase-3, caspase-11 (that controls caspase-1 activation), protein p53, or interleukin IL-1β (activated by caspase-1), and it cannot be suppressed by anti-apoptosis proteins Bcl-2 and Bcl-X_L [5]. Invasion proteins (IpaB and SipB) activate caspase-1. The activation mechanism has not yet been elucidated [5, 6]. The question to raise is whether proteins IpaB and SipB, like mitochondrial serine protease Omi/HtrA2, neutralize the effects of microbial inhibitors of caspases (IAP, Inhibitor of Apoptosis Protein)? Apart from IpaB and SipB, micromolar concentrations of the channel-forming protein of the outer membrane of *Pseudomonas aeruginosa* cells induce the apoptosis of epithelial cells [7].

The intracellular location of a pathogen is not a prerequisite for induction of apoptosis. The pathogen can inject toxic proteins into a target cell upon contact with it. This type of delivery of cytotoxic proteins requires cell–cell contact and is referred to as the type III secretion mechanism [8]. It is used by *Ps. aeruginosa* for induction of apoptosis in macrophages, epithelial cells [9, 10], and neutrophils [11].

Ps. aeruginosa is an aerobic bacterium that is widespread in nature. It is so modest with respect to its nutrient requirements that it can grow in distilled water. It switches over to the denitrification mode of metabolism under anaerobic conditions, using NO_3^- as an electron acceptor. This gram-negative bacterium that represents one of the major human opportunistic pathogens (only *Staphylococcus aureus* and *Escherichia coli* are still more important in this respect) produces the antibiotic pyocyanine, a blue phenazine pigment that is responsible for the characteristic blue puss seen in wounds infected with this bacterium.

The activation of procaspase-3 and the apoptosis of macrophages and mast cells are induced by the mixture of azurin and cytochrome c_{551} secreted by *Ps. aeruginosa* cells into the growth medium [4]. Azurin is a low molecular weight (14 kD) copper-containing protein that is presumably involved in dissimilatory nitrate reduction as an electron carrier operating between cytochrome c_{551} and nitrite reductase [12, 13].

Subsequent studies revealed [14] that tumor cells (cells of the human melanoma UISO-Mel-2) *in vitro* take up azurin that activates caspase-9 and caspase-3 and induces apoptosis that is detectable by DNA fragmentation. The azurin effect manifests itself in protein p53-expressing melanoma cells. Azurin cytotoxicity is much less significant in p53-null mutant melanoma cells. Only redox-active azurin possesses apoptosis-inducing properties. Redox-incompetent mutant azurin fails to elicit apoptosis in melanoma cells.

Azurin is predominantly located in the cytoplasm and the nucleus of tumor cells. It is detectable only in the cytoplasm of p53-null mutant cells. Accordingly, the intracellular transport of azurin is protein p53-dependent [14]. Moreover, azurin forms a complex with p53.

Protein p53 is the central component of the system that secures instantaneous elimination of damaged and potentially dangerous cells (reviewed in [15]). p53 is a transcription factor. This is its main function, which is performed by the protein in the cell nucleus. Protein p53 is activated upon cell disruption. Activated p53 coordinates the restoration of impaired cell structures or induces apoptosis if the restoration is impossible. Disruption of the *p53* gene or of the p53 activity-regulating genes results in uncontrollable accumulation of genetic defects, malignant transformation of the cells involved, tumor development, and the organism's death. Protein p53 is highly labile. Its pool is constantly renewed; protein p53 is resynthesized and undergoes ubiquitin-dependent degradation in proteosomes. The p53 content is low (near the threshold of detection) in normal cells.

Its stability depends on protein–protein interactions. Complexing with azurin enhances p53 stability and thereby increases its content in the cytoplasm, mitochondrial, and cell nucleus fractions [14]. It is the stabilization of protein p53 and the increase in its content resulting from complexing with azurin that apparently provide for the cytotoxicity of azurin. An increase in the content of proapoptotic protein Bax in mitochondria is correlated with an increase in the intracellular p53 content. This causes the liberation of cytochrome *c* from the intermembrane space of mitochondria and engages the mechanisms of caspase-9 and caspase-3 activation. The azurin effect manifested itself, apart from tumor cell cultures, in mice with xenotransplanted human melanoma. Intraperitoneal azurin injections caused the apoptosis of tumor cells and cancer regression in thymusless (nude) mice [14]. It is an open question how the injected azurin reached the xenotransplant cells.

The cytolytic effect of *Ps. aeruginosa* is not selective. As noted above, *Ps. aeruginosa* induces apoptosis in macrophages, epithelial cells, neutrophils, and tumor cells. We should expect obligatorily anaerobic bacteria to be more selective, because anaerobic areas form in tumor tissues only. What is the mechanism of the destructive effect of anaerobes? Judging by the data presented in [5, 6], the mechanism is based on apoptosis. Different anaerobes may use different apoptosis-inducing strategies. Research in this field can be very fruitful.

ANTICANCER VACCINES BASED ON BACTERIAL VECTORS

As noted in [16], a number of mechanisms enable tumors to escape the effects caused by immune responses. Rapidly growing tumors are not infiltrated by lymphocytes, i.e., no inflammation occurs in the tumor zone, and antigen-presenting cells (APC) are neither recruited nor activated. Tumor cells release immunosuppressive factors, e.g., transforming growth factor β and interleukin-10. In addition, dying tumor cells undergo apoptosis that is not accompanied by the release of antigens into the extracellular matrix. Tumor cells *per se* lack APC properties and, therefore, cannot prime (sensitize) T lymphocytes. The transfer of antigenic determinants from tumor cells to professional APC, mainly to dendrite cells and to a lesser extent to macrophages, is a prerequisite for the activation of cytotoxic $CD8^+$ T-lymphocytes (T killers).

One approach to targeting APC *in vivo* is to use attenuated bacterial vectors selectively propagating within dendrite cells and macrophages. A promising candidate for this role is *Listeria monocytogenes*. This bacterium can be used as a vaccine carrier transferring into APC tumor antigens that can undergo processing, i.e., cleaving into peptide fragments binding to molecules of the class I or class II main histocompatibility complex [17, 16].

L. monocytogenes is a gram-positive bacterium and a facultative intracellular parasite. Meningitis, sepsis, and endocarditis are the most frequent manifestations of lysteriosis. The immune response to the *L. monocytogenes* invasion is accompanied by the production of cytokines (e.g., interleukin-12) and mediators (e.g., NO) that potentiate antigen presentation. Attenuated recombinant transgenic *L. monocytogenes* strains that carry tumor antigens proved to be efficient anticancer vaccines [18, 19]. *Salmonella* representatives are also efficient in transferring antigens [20].

Recombinant vaccines can be used to secure the readiness of the immune system of an organism to repulse an assault of malignantly transformed cells [16]. Such a situation can arise after the surgical removal of a tumor if the patient still shows residual symptoms of the disease (vaccination aimed at preventing a relapse).

SUPPRESSION OF TUMOR ANGIOGENESIS BY MICROORGANISMS

The growth of solid tumors is accompanied by angiogenesis, i.e., the formation of new blood vessels that supply a tumor with nutrients. Tumors produce a number of factors. Some of them stimulate angiogenesis, while other factors inhibit it [21].

Infecting mice with *Toxoplasma gondii* blocked the development of the non-immunogenic melanoma B16F10 [22]. *T. gondii*, a protozoan of the *Sporozoa* group, causes toxoplasmosis. Animals including domestic species, e.g., cats, and humans suffer from this disease. *T. gondii* is an obligatory intracellular parasite that inhabits macrophages and parenchyma cells of the liver, lungs, brain, and other organs.

The protozoans *T. gondii* and *Besnoitia jellisoni*, like the bacteria *L. monocytogenes*, *Corynebacterium parvum*,

and *Mycobacterium bovis*, are capable of activating macrophages. Activated macrophages become nonspecific killers of tumor cells *in vitro*. The process involves the mediators NO (produced by inducible NO synthase) and tumor necrosis factor TNF-α (see [22] and references cited therein). A similar effect is produced by the antituberculosis vaccine BCG (vaccine strain *M. bovis* bacillus Calmette–Guerin) widely used for treating urinary bladder cancer [23]. However, the suppression of melanoma development in *T. gondii*-infected mice was independent of NO production by macrophages and of the cytokines TNF-α and interleukin-12, cytotoxic T lymphocytes, or natural killers [22].

These data indicate that tumor suppression is based on an alternative mechanism not involving cytotoxicity. The *T. gondii* infection results in the suppression of angiogenesis and, as a consequence, in the arrest of tumor development [22]. The *T. gondii*-dependent inhibition of tumor angiogenesis is apparently due to the release of soluble anti-angiogenic factors. Elucidating their chemical nature would enable us to extend the list of medicines used for treating cancer. It was earlier shown that bacterial polysaccharides isolated from the culture fluid of *Serratia marcescens* exert an inhibitory influence on tumor angiogenesis [24].

In addition to *T. gondii*, other protozoans also exhibit anticancer activity, although their mechanism of action has not yet been clarified. An antagonistic relationship between malignant tumor growth and Chagas disease (caused by *Tripanosoma cruzi* of the *Flagellates* group) was revealed as early as 1931 [25]. This made it possible to develop two anticancer preparations from *T. cruzi*: cruzin (Russia) and its analog tripanose (France). Antitumor activity is characteristic of trypanosomes that dwell in tumor cells as their parasites and of dead *T. cruzi* cells (see [26] and the references cited therein for more detail).

The data considered here indicate that bacteria and protozoans (predominantly pathogens) produce antitumor effects. A number of pathogenic viruses also cause similar effects [27].

A simple explanation of these data is as follows [27]. Pathogens propagate in the macroorganism and destroy the cells and tissues affected by them either directly or using the immune system of the host. The destructive potential of pathogens is not selective, and we can put it to use by aiming it at tumor cells. This explanation does not apply, however, if we deal with the nonpathogenic, symbiotic species *Bifidobacterium bifidum* or the free-living *Clostridium acetobutylicum* [1]. Probably, there are mechanisms that are responsible for the selective impact of microbes on tumor cells. Of special interest in this respect is *B. bifidum*. The mechanism of its antitumor effects should be clarified. It is also necessary to test other nonpathogenic symbiotic and free-living microorganisms.

In the light of the new options based on microbial therapy of cancer, we should regard symbiotic and pathogenic microorganisms as powerful agents that can help us hold in check the development of malignant tumors. It is essential that the equilibrium between a macroorganism and its microflora should not be disturbed.

This study was supported by the Russian Foundation for Basic Research (grant No. 01-04-48356).

REFERENCES

1. Jain, R. K., and Forbes, N. S. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 14748-14750.
2. Dang, L. H., Bettegowda, C., Huso, D. L., Kinzler, K. W., and Vogelstein, B. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 15155-15160.
3. Samuilov, V. D., Oleskin, A. V., and Lagunova, E. M. (2000) *Biochemistry (Moscow)*, **65**, 873-887.
4. Zaborina, O., Dhiman, N., Chen, M. L., Kostal, J., Holder, I. A., and Chakrabarty, A. M. (2000) *Microbiology*, **146**, 2521-2530.
5. Hilbi, H., Moss, J. E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., Flavell, R. A., Yuan, J., Sansonetti, P. J., and Zychlinsky, A. (1998) *J. Biol. Chem.*, **273**, 32895-32900.
6. Hersh, D., Monack, D. M., Smith, M. R., Ghori, N., Falkow, S., and Zychlinsky, A. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 2396-2401.
7. Buommino, E., Morelli, F., Metafora, S., Rossano, F., Perfeto, B., Baroni, A., and Tufano, M. A. (1999) *Infect. Immunol.*, **67**, 4794-4800.
8. Frank, D. W. (1997) *Mol. Microbiol.*, **26**, 621-629.
9. Hauser, A. R., and Engel, J. N. (1999) *Infect. Immunol.*, **67**, 5530-5537.
10. Coburn, J., and Frank, D. W. (1999) *Infect. Immunol.*, **67**, 3151-3154.
11. Dacheux, D., Attree, I., Schneider, C., and Toussaint, B. (1999) *Infect. Immunol.*, **67**, 6164-6167.
12. Van de Kamp, M., Silverstrini, M. C., Brunori. M., van Beeumen, J., Hali, F. C., and Canters, G. W. (1990) *Eur. J. Biochem.*, **194**, 109-118.
13. Vijgenboom, E., Busch, J. E., and Canters, G. W. (1997) *Microbiology*, **143**, 2853-2863.
14. Yamada, T., Goto, M., Punj, V., Zaborina, O., Chen, M. L., Kimbara, K., Majumdar, D., Cunningham, E., Das Gupta, T. K., and Chakrabarty, A. M. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 14098-14103.
15. Chumakov, P. M. (2000) *Biochemistry (Moscow)*, **65**, 28-40.
16. Paglia, P., and Guzman, C. A. (1998) *Cancer Immunol. Immunother.*, **46**, 88-92.
17. Kaufmann, S. H. E. (1993) *Annu. Rev. Immunol.*, **11**, 129-163.
18. Pan, Z. K., Lazenby, A., Pardoll, D., and Paterson, Y. (1995) *Nature Med.*, **1**, 471-477.
19. Paglia, P., Arioli, I., Frahm, N., Chakraborty, N., Colombo, M. P., and Guzman, C. A. (1997) *Eur. J. Immunol.*, **27**, 1570-1575.
20. Hess, J., Gentschev, I., and Miko, D. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 1458-1463.

21. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) *Cell*, **88**, 277-285.
22. Hunter, C. A., Yu, D., Gee, M., Ngo, C. V., Sevignani, C., Goldschmidt, M., Golovkina, T. V., Evans, S., Lee, W. F., and Thomas-Tikhonenko, A. (2001) *J. Immunol.*, **166**, 5878-5881.
23. Alexandroff, A. B., Jackson, A. M., O'Donnel, M. A., and James, K. (1999) *Lancet*, **353**, 1689-1694.
24. Algire, G. H., Legallais, F. Y., and Park, H. D. (1947) *J. Natl. Cancer Inst.*, **8**, 53.
25. Roskin, G. I., and Exemplarskaja, E. (1931) *Zeitschr. Krebsforsch.*, **34**, 628-645.
26. Kallinikova, V. D., Matyokin, P. V., Ogloblina, T. A., Leikina, M. I., Kononenko, A. F., Sokolova, N. M., and Pogodina, L. S. (2001) *Izv. Akad. Nauk. Ser. Biol.*, No. 3, 299-311.
27. Kirn, D. H. (2000) *J. Clin. Invest.*, **105**, 837-839.