ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 6, pp. 597-606. © Pleiades Publishing, Inc., 2006.
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REVIEW

Eukaryotic Expression Vectors and Immunoconjugates for Cancer Therapy

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Received September 19, 2005
Revision received October 21, 2005

Abstract—This review considers ways to address specificity to therapeutic targeted anticancer agents. These include transcriptional activation of tissue- and tumor-specific promoters in eukaryotic expression vectors and use of antitumor-directed immunoconjugates. The review deals with analysis of strategies used for selection of targeted promoters and examples of antibody fusion proteins exhibiting antitumor activity. A new direction in antitumor treatment pooling together methods of gene therapy and antibody therapy has appeared. This direction is based on the development of vectors encoding secreted forms of immunoconjugates. After vector introduction into a cell, the latter is capable of synthesizing and secreting antibody fusion protein composed of a therapeutic anticancer agent and antibody specifically targeted to cancer cells.

DOI: 10.1134/S0006297906060022

Key words: gene therapy, fusion protein, secreted protein, immunoconjugate, anticancer agent

The design of vectors promoting tumor suppression is one of the intensively developed directions of gene therapy. Controlled therapeutic gene delivery to malignant tissues and regulation of transgene expression play the most important role in the targeted gene therapy of cancer. Viral and non-viral vectors are used for delivery of therapeutic genes. The viral gene delivery vectors include replication-deficient retroviruses, adenoviruses, and some other viruses. Retroviral vectors are able to infect dividing cells only. Adenoviral vectors can transduce both dividing and non-dividing cells. They are not integrated into the host cell genome and therefore provide only short-term gene expression [1, 2]. Plasmid DNA is not integrated in the host cell genome and also provides only short-term gene expression [3]. It may be directly transfected into cells by direct injections [4], in a complex with liposomes [5], or by ballistic transfection into the target organ [6]. Exogenous regulation of duration and level of therapeutic gene expression is determined by the presence of inducible promoters in vectors; such promoters can be activated by ionizing radiation [7], estrogens [8], and heavy metal ions [9]. The constructed tetracycline-controlled gene expression system (Tet-Off and Tet-On) has several important advantages compared with other expression systems. These include flexible regulation, high inducibility, and rapid response [10, 11]. This expression system is controllable both *in vitro* and *in vivo* [12].

The strategy of selection of therapeutic genes is based on understanding of the molecular biology of cancer, complex interactions between tumor cells, and the immune system. Constructions for tumor suppression employ genes whose expression induces cell death or stimulation of the immune response. Suicide gene therapy of malignant tumors requires effective gene transfer and highly selective gene expression [13]. Targeted delivery of the therapeutic agent may be realized by using tissue specific/tumor specific promoters and also by antibodies. Various vectors and immunoconjugates have now been developed for cancer therapy.

This review summarizes data on eukaryotic expression vectors constructed for selective elimination of cancer cells. We describe here two main strategies of targeted delivery of the therapeutic agent. The first strategy employs tissue/tumor specific promoters, whereas the second one uses antibodies. It is possible to combine these strategies by designing expression vectors encoding secreted hybrid proteins that consist of an antibody and a therapeutic protein.

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STRATEGY FOR SELECTION OF TARGETED PROMOTERS

In certain tissues targeted vectors induce transcriptional activation of tissue/tumor specific promoters resulting in transcription of the mRNA of the therapeutic gene, which causes selective elimination of cancer cells. However, the expression of the transgene may cause a toxic effect both in malignant and normal tissues and this is one of the main limitations of the use of such tissue-specific promoters. So, such promoters are used mainly for therapy of organs and tissues that are not "critical" for survival (e.g., prostate, melanocytes, thyroid gland). In the case of vitally important organs or tissues, the transgene has to be delivered directly to tumor cells by means of retroviral vectors. For example, the tissue-specific promoter of the albumin gene is delivered into hepatocytes [14-16]. Retroviral vectors are less effective in infecting non-dividing, slowly proliferating normal hepatocytes than in infecting liver cancer cells [17]. The following promoters are used for tissue-specific expression of a therapeutic gene: surfactant protein B promoter is used for expression in alveolar cells type II and bronchial cells [18, 19]; tyrosinase promoter is used for expression in melanocytes [20-22], and thyroglobulin promoter and *OSP-1* promoter are used for expression in thyroid gland [23-25] and ovary [26, 27], respectively.

In the ideal case, tissue-specific promoter would also be tumor-specific promoter. The examples of the tumor specific-promoters of genes that are highly active in cancer cells and exhibit low or no activity in normal cells include promoter of the gene encoding prostate specific antigen (PSA) [28-31] and promoter of calcitonin encoding gene, which are highly active in prostate cancer cells [32]. Telomerase is highly active in most cancer but not in normal cells [33, 34] and promoter of catalytic subunit of the human telomerase gene is used for targeted gene therapy of ovarian cancer cell line [35]. Other examples are: promoter of gene encoding secretory leukoprotease inhibitor (SLPI), which is constitutively expressed in epithelial carcinoma cells [36]; promoter of the gene encoding human chorionic gonadotropin (hCG-β), which is active in testicular cancer [37, 38]; MUC1/DF3 is active in breast and pancreas cancers [39, 40], stomach cancer [41], and cholangiocarcinomas [42]; HER2/neu is active in breast cancer [43, 44] and ovary cancer [45].

Thyroid-specific transgene expression. Thyroglobulin gene promoter (TG). Thyroglobulin is a soluble glycoprotein involved in synthesis and storage of thyroid hormones. The promoter of the gene encoding thyroglobulin is used for tissue-specific expression of therapeutic genes in thyroid cell lines. Almost 100% of thyroid gland carcinoma cells and only 5% of control cells died from ganciclovir during infection of cells with adenovirus expressing HSV-tk (*Herpes simplex* thymidine kinase) under control of the thyroglobulin promoter [23]. Thymidine kinase-deoxyribonucleoside kinase (Tk) phosphorylates thymidine, deoxycytidine, deoxyuridine, and also antiviral and antitumor nucleoside analogs [46]. Tk phosphorylates nontoxic GCV, the nucleoside analog, to the nucleotide (GCV-P). Addition of two phosphates to GCV-P catalyzed by endogenous kinases results in formation of ganciclovir triphosphate (GCV-P-P-P). GCV-P may also enter neighboring cells where it is phosphorylated by endogenous kinases. Ganciclovir triphosphate can be incorporated into DNA, but DNA polymerase cannot replicate DNA containing the ganciclovir nucleotide analog. As a result, the mitosis is interrupted and the cell is killed. Since GCV-P can be transported into neighboring cells, it is not necessary to deliver such vector into each cancer cell that should be killed [47]. Adenovirus containing *HSV-tk* under control of tandemly-repeated minimal thyroglobulin promoter (Ad2xTG-tk) was 10-30 times more effective in killing normal rat thyroid gland cells (FRTL5) producing thyroglobulin and follicular thyroid carcinoma cells (FTC-133) than adenovirus with minimal TG promoter (AdTG-tk). FTC-133 tumor-bearing nude mice, when treated with Ad2xTG-tk, exhibited more pronounced tumor reduction than animals carrying these carcinoma cells treated with AdTG-tk [48].

Prostate-specific transgene expression. Prostate-specific antigen (PSA) gene promoter. PSA is an intracellular glycoprotein of 34 kD specifically synthesized by prostate cells. Its expression is regulated by androgens. Serum PSA level increased in patients with prostate cancer. The *PSA* gene promoter can induce reporter gene expression in cell lines (LNCaP) producing PSA but not in control cell lines (DU145 and PC-3) that do not express PSA [28]. A complex adenoviral vector has been constructed to combine cell-specificity and high level of regulation of transgene expression. The adenoviral vector containing *PSA* promoter/enhancer caused 20-fold increase of therapeutic gene expression in LNCaP cell line but not in *PSA*-negative control cell lines. Tandem duplication of *PSA* enhancer caused 50-fold increase of the therapeutic gene expression; expression from the enhancer construct was increased 100-fold above basal levels when induced with the androgen dihydrotestosterone [49]. Differential expression of protein product of the requested gene in target tissues is the central concept of gene therapy. One of approaches used for differential expression of protein product consists in use of tissue-specific promoter for induction of therapeutic gene transcription. In different species of cancer cells, there is impairment of the tumor suppressor *p53* gene. Mutations of *p53* are of the most frequent changes typical for growth and so the introduction of intact gene into tumor cells will cause their death [50, 51]. The *PSA* promoter or promoter/enhancer were tested for tissue-specific expression of the therapeutic *p53* gene. The experiments revealed that *p53* under control of *PSA* promoter or *PSA* promoter/enhancer cassette effectively suppressed growth of PSA-producing prostate cancer cell

line (LNCaP) but was ineffective in cell lines that did not produce PSA (DU145, PC-3). Transcription of *p53* under control of *PSA* promoter/enhancer more effectively suppressed LNCaP cell line growth *in vitro* [52]. Lentiviral vector containing tissue-specific *PSA* promoter was used for delivery of the diphtheria toxin A (DTA) gene to prostate cancer cells. Effectiveness of killing of human prostate cancer cells *in vivo* was shown using nude mice bearing LNCaP xenografts. A single injection of the *DTA* lentiviral vector into LNCaP prostate tumors caused complete eradication of approximately 75% of the tumors [53]. Vector ADV.ARR(2)PB-iCasp9 was constructed for expression in prostate cancer cells of inducible caspase-9 (iCaspase-9) under control of the prostate-specific promoter *ARR(2)PB* [54]. Caspases are cysteine proteases that play an important role in cleavage of various cell proteins [55]. These enzymes are involved in programmed cell death (apoptosis) [56]. In *in vivo* experiments ADV.ARR(2)PB-iCasp9 induced apoptosis in tumor LNCaP cells [54]. The apoptotic process involves caspases of at least two classes: initial caspases (caspase-2, caspase-8, caspase-9, caspase-10) and effector caspases (caspase-3, caspase-6, caspase-7) [56-61]. For example, conversion of procaspase-8 into activated caspase-8 causes activation of caspase-3. Caspase-3 also exists in cells as an inactive proenzyme, which contains N-terminal prodomain, large, and small subunits. During the activation process, the precursor molecule is specifically cleaved at a site containing several amino acid residues (Ile-Glu-Thr-Asp) between domains; this results in prodomain removal followed by the re-association of the large and the small subunit into a heterodimer [62]. The tetracycline-transactivator gene was placed under control of prostate-specific *ARR(2)PB* promoter, and mouse Tnfsf6–GFP fusion gene under the control of the tetracycline responsive promoter. *Tnfsf6* encodes FASL, an integral membrane protein of 40 kD that belongs to a tumor necrosis factor family. The latter may be released into extracellular medium and act as a cytokine that binds to specific receptor and triggers apoptotic signal. *FASL–GFP* expression was essentially restricted to prostate cancer cells, in which it can be regulated by doxycycline. Increase in *FASL–GFP* expression correlated with greater induction of apoptosis in prostate cancer LNCaP cells [63].

Melanocyte-specific transgene expression. Tyrosinase, also known as *ortho*-diphenol oxidase, has been found in almost all animals and plants. The enzyme catalyzes the oxidation of tyrosine to 3,4-dihydroxyphenylalanine during biosynthesis of melanin pigments in melanocytes. Human and murine melanoma cell lines are characterized by high level of reporter gene expression under control of the promoter, which contains 5′-flanking sites of the tyrosinase and tyrosinase-binding protein 1 genes. Effectiveness of its functioning has been demonstrated during tissue-specific expression of *HSV-tk/GCV* [20], interleukin-2, and interleukin-4 [64]. Therapeutic genes are delivered to cancer cells under control of tyrosinase promoter using retroviral [65], adenoviral vectors [21], and conditionally replicating adenoviral vectors with synthetic melanocyte-specific tyrosinase enhancer/promoter construct (Tyr2E/P) [66, 67]. Recombinant adeno-associated viral particles (rAAV) containing the tissue-specific human melanoma inhibitory activity gene promoter (*hMIA*) were combined with four copies of the enhancer element of the murine tyrosinase gene for melanoma therapy. Transient cell transduction with viral particles containing the therapeutic *HSV-tk* gene under control of enhancer/*MIA* promoter resulted in selective suppression of melanoma cell growth. Experiments on nude mice with transplanted human melanoma revealed tumor reduction [68]. The adenoviral vector in which four copies of murine tyrosinase enhancer element (*TE*) were combined with the tyrosinase promoter (*TP*) exhibited 2000-fold increase in luciferase reporter activity in melanoma cells compared with non-melanoma cells. For tissue-specific expression of E1A in the melanoma cells, *E1A* was placed under *TETP*. (Internal adenoviral enhancer/promoter (EP) and E-3 site were removed.) The resulting AdDeltaEP-TETP vector was replicated 50 times more effectively in tyrosinase-positive melanoma cells (SK-Mel23) than in non-melanoma cells. Injection of AdDeltaEP-TETP into xeno-transplanted melanomas, but not into HeLa-derived tumors, resulted in tumor regression in nude mice [69]. Adenoviral E1A proteins are transcription regulators exhibiting anti-oncogenic and cell transforming properties [70]. Comparison of amino acid sequences of E1A proteins from viruses belonged to different serotypes revealed three conservative regions (CR) [71-73]. CR3 exhibits potent trans-activated function, whereas CR1 and CR2 are required for oncogenic activity of E1A proteins [74]. E1A proteins form complexes with certain cell proteins playing an essential role in cell growth and differentiation. It is suggested that E1A binding to these proteins alters (or inhibits) their normal functioning in the cell [75]. For example, the CR2 region is responsible for binding to retinoblastoma pRb protein [76] and also for immortalization required for oncogenic activity of E1A proteins. Rb protein binds transcription factor E2F and prevents its transcription activation of genes required for transition of cells into S phase, the phase of DNA replication. Binding of the CR2 region with pRb promotes release of E2F. E1A is a transcription regulator repressing HER2/neu overexpression [73, 77]. Deletion of the CR2 region did not influence the ability of E1A to repress *HER2/neu* gene expression. This observation was used for the construction of a mini-E1A mutant lacking the CR2 region; this mutant is a safer therapeutic agent exhibiting selective tumor suppressive activity [78]. The nucleotide sequence of the *E1A* gene is often used in constructs tested for tumor suppression activity.

The adenoviral-based construction Ad-Tyr-Epo/TNFR1 employs antitumor activity of tumor necrosis

factor (TNF-α), which may be selectively triggered for tumor killing without its systemic toxic effects. The gene of constitutively active TNF receptor of 55 kD, which can kill cells even without its ligand [79], was placed to the adenoviral vector under control of tyrosinase gene promoter. Expression and receptor functioning was specifically restricted to melanoma cells; this caused apoptosis via activation of the caspase cascade [80]. Many strategies of cancer gene therapy imply indirect killing of tumor cells by stimulating host immune response using various cytokines such as tumor necrosis factor, interleukins, chemokines, colony stimulating factors, and interferons. The effect of cytokines involves the so-called cytokine cascade when cytokine action on one cell causes formation of other cytokines in this cell. TNF-α is a cytokine responsible for induction of necrotic and apoptotic cell death [81-83].

Uterine cervical transgene expression. *MN/CA9* promoter. The *MN/CA9* gene encodes a transmembrane glycoprotein that is an isoenzyme of the carbonic anhydrase family. Initially it was identified in human cervical carcinoma cell line and HeLa cells. Its overexpression was observed in malignant carcinomas [84, 85]. Taking into consideration high level of MN/CA9 protein in uterine cervical carcinoma cells, the adenoviral vector employing *MN/CA9* promoter (Ad-MN/CA9-E1a) has been constructed. It can be replicated only in cells expressing MN/CA9. Injection of Ad-MN/CA9-E1a vector into a tumor effectively inhibited growth of xeno-transplanted HeLa cells in nude mice [86].

Tumor-specific transgene expression. Human telomerase. Telomerase is a ribonucleoprotein complex that adds telomere repeats $(TTAGGG)_n$ to the chromosome ends and thus plays an important role in immortalization of cells. Telomerase consists of two major components: catalytic subunit (hTERT) and RNA subunit (hTR) [87]. Telomerase is highly active in ~90% of human malignant tumor, whereas in normal somatic tissues its activity is low (or the enzyme is not expressed) [88, 89]. Expression of hTERT subunits was found only in cancer or embryonic cells. The *hTERT* promoter exhibits potent transcription activity in most cancer cell lines; thus it may be an optimal promoter for realization of a univector gene therapeutic approach based on high tumor selectivity of this promoter [90]. Recombinant adenovirus (Ad-hTTK) containing *hTERT* promoter controls expression of the therapeutic *HSV-TK* gene and can be used for gene therapy of gynecological malignant tumors [35]. The expression vector (hTERT/rev-caspase-6) containing the gene of constitutively active caspase-6 (rev-caspase-6) under control of the *hTERT* promoter induced apoptosis in hTERT-positive cells of malignant gliomas. Expression of rev-caspase-6 induced apoptosis, which did not depend on the activity of the initial caspases [91]. Expression of *E1A* under transcription control of *hTERT* promoter was effective for telomerase-dependent replication of adenoviral vector in tumor cells. This vector was tested using tumor cell lines CaCo2, HeLa, AGS, Huh7, and Hep3B. Expression of hTERT-Ad in nude mice Huh7-xenografts caused significant inhibition of tumor growth [92]. Bicistronic adenoviral vector (Ad/gTRAIL), expressing a fused protein under *hTERT* promoter caused apoptosis in cancer but not in normal liver cells. This protein consists of green fluorescent protein (GFP) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), the apoptosis-inducing ligand which is related to tumor necrosis factor [93]. The recombinant adenovirus containing HSV-tk under control of *hTERT* promoter is a potentially interesting vector for gene therapy of non-differentiated thyroid carcinoma [94].

Tumor-specific transgene expression in breast, stomach, pancreatic cancer cells, and cholangiocarcinomas. *MUC1/ DF3* gene promoter. The *MUC1* gene encodes a high molecular weight mucin-like protein that provides a protective layer on the surface of epithelial cells; it contains a membrane-bound extracellular subunit [95]. *MUC1* overexpression has been observed in breast cancer and in cholangiocarcinomas. Cell transfection with constructs with nucleotide sequence of 2.9 kb 5′-flanking the *MUC1* sequence caused expression of the reporter gene encoding chloramphenicol acetyl transferase (CAT) in pancreas and breast cells, but not in non-epithelial HT-1080, SK23, and HTB96 cell lines. Maximal *CAT* expression was found in ZR-75 (breast cancer cells) and HPAF (pancreatic cancer cell) but only in the presence of the 743 bp 5′-flanking nucleotide sequence of *MUC1* [39]. Use of amplified *MUC1/DF3* promoter in mucin-positive cell lines caused 590-fold increase in fused Gal4VP16 protein expression compared with expression of the same gene placed under control of the *CMV* promoter [96]. The adenoviral vector (AdMUC1-hSSTR2) carrying amplified *MUC1* promoter and expressing human somatostatin receptor of subtype 2 (hSSTR2) induced apoptosis in pancreatic cancer cells [40].

Tumor-specific transgene expression in breast, pancreatic, and ovarian cancer cells. *HER2/neu* promoter. The *HER2/neu* gene, also known as *erbB2*, encodes epidermal growth factor receptor type 2, a transmembrane protein of 185 kD exhibiting tyrosine kinase activity [97, 98]. In human epithelial cells, *HER2/neu* expression is low. *HER2/neu* overexpression results in malignant transformation of epithelial cells [99]. *HER2/neu* promoter differs from promoter of the gene encoding epidermal growth factor receptor type 1 (*HER1*). For example, *HER2/neu* lacks GC-elements typical for *HER1* promoter. Transcription of *HER2/neu* can be regulated by a mechanism involving a TATA-box and some non-identified regulatory elements [43, 100]. *HER2/neu* promoter can control the tissue-specific expression of reporter gene *GFP* in ovarian cancer cell lines [45]. Expression of the therapeutic gene encoding cytosine deaminase under control of the *HER2/neu* promoter results in death of

HER2/neu-positive cells [101]. The construct of minimal *HER2/neu* promoter containing a fragment of 251 bp (−213/+38) specifically induced transcription of a reporter gene in breast cancer cells. *HSV-tk* gene placed under control of this promoter increased sensitivity of breast cancer cells to GCV and suppressed growth of these cells in nude mice [102].

Ovary-specific transgene expression. *OSP-1* promoter. Ovary-specific promoter *OSP-1* has a sequence of 462 bp that is a part of a retroviral-like element specifically expressing in rat ovary [26]. The effectiveness of its functioning was demonstrated during *HSV-tk* expression under control of this promoter [27].

Dual-specificity promoter systems. Combined use of various strategies for selective treatment of cancer cells attracts much attention of researchers. A new dual-specificity promoter system for tissue-specific transcription under control of tissue-specific promoter inducing expression of the therapeutic gene was constructed for specific expression of the therapeutic gene in cancer cells and reduction of side effects. Applicability of this system was demonstrated using lung cancer cells. This system is called TTS, the *TTF1* gene under control of *hTERT* promoter and surfactant protein A1 promoter. Promoter activity of this TTS system was much higher than in other cancer cells and also in normal lung cells [103]. The combination of cyclin A and tyrosinase promoters was used for transgene expression in proliferating melanoma cells. Expression of the therapeutic *TNF-α* gene under control of the dual-specificity promoter system was sufficient for manifestation of cytotoxic effects [104]. Subsequent studies in this direction resulted in development of the dual-specificity promoter system combining tissue-specificity with regulation of the cell cycle. The chimeric transcription factor (Gal4/NF-Y), which consists of the transactivation domain NF-Y and DNA-binding Gal4 domain, is activated by the tissue-specific promoter. The transcription factor Gal4/NF-Y can bind to the second promoter, which consists of cyclin A minimal promoter, to multiple Gal4-binding sites and replace normal UAS-activating sequences (upstream activating sequence). Expression of this system was 50-times more specific and the regulation of cell cycle was more than 20-times higher in proliferating melanoma cells than in control cells [105].

Simultaneous administration of two vectors into tumor cells. Simultaneous administration of two therapeutic vectors may effectively suppress a tumor. Secretory leukoprotease inhibitor (SLPI) is actively expressed in almost all lung tumors (NSCLCs), but not in other tumor types. The adenoviral vector with dual expression cassette (AdSLPI.E1AdB) was constructed for specific gene therapy of NSCLC. In this expression cassette *E1A* was under the promoter of the *SLPI* gene positioned before *E1B-19K*, which was placed under control of cytomegalovirus (CMV) promoter; the latter can be selectively replicated in NSCLC cells. Use of AdSLPI.E1AdB significantly inhibited proliferation of NSCLC cells *in vitro*. Direct injection of AdSLPI.E1AdB into A549 and H358 tumor xenografts in nude mice resulted in significant reduction of tumors in these mice compared with control (A549, 57%, $P < 0.02$; H358, 67%, $P < 0.03$). The authors also evaluated AdSLPI.E1AdB and AdCMV.NK4 containing a gene encoding NK4 protein, which exhibits potent anti-angiogenic activity. Injection of the two vectors AdSLPI.E1AdB and AdCMV.NK4 into H358 tumor resulted in more potent reduction in tumor growth compared with the effect of one vector [106]. Administration of the recombinant adenovirus (AdTCPtk) expressing *HSV-tk* gene under control of modified *TCP* promoter into cell lines of medullar thyroid carcinoma (MTC) caused significant cytotoxic effect *in vitro* after treatment of cells with ganciclovir (GCV). Simultaneous injection of AdTCPtk/GCV and AdTCPmIL-12 (mIL-12 is mouse interleukin-12) provided more effective suppression of tumors in WAG/Rij rats than independent injections of AdTCPtk/GCV or AdTCPmIL-12 [107]. The *HSV-tk* gene placed under control of thyroglobulin is used for therapy of thyroid carcinoma. However, lowly differentiated or anaplastic carcinomas cannot express thyroglobulin due to loss of transcription factors (thyroid transcription factor-1 (TTF-1), TTF-2, or Pax-8) interacting with the thyroglobulin promoter. Co-transduction of adenoviral vectors AdTTF-1 and AdTGTK caused death of 90% of cells of BHP15-3 cell line (TTF-1(−)/TTF-2(−)/Pax-8(−)/TG(−)), 95% of cells of rat normal thyroid cell lines (FRT), and also cells of lowly differentiated thyroid cell lines BHP7-13 and BHP18-21v (TTF1(−)/TTF-2(−)/Pax-8(+)/TG(−)), which do not express the thyroglobulin (*TG*) gene [108].

Although many vectors expressing therapeutic genes in the target cell under control of tissue and tumor-specific promoters are now available, studies in this direction continue. Delivery systems and promoters are being modified and the spectrum of therapeutic genes for the development of optimal constructions for cancer therapy is being extended.

USE OF ANTIBODIES FOR DELIVERY OF THERAPEUTIC AGENTS

The use of antibodies for delivery of therapeutic agents to tumor cells is another direction in cancer therapy. Full-sized and mini-antibodies are employed for this purpose. Mini-antibodies consist of scFv-, Fab-, and $F(ab)_2$-fragments of the immunoglobulin molecules with removed constant fragment Fc [109, 110]. High specificity and affinity of antibody interaction with its antigens are employed for high precision delivery of therapeutic agents to target cells expressing these antigens, tumor markers [111, 112]. For example, immunotoxins consisting of antibodies fused with toxins have been developed. The

toxins included in gene therapeutic constructions cause tumor regression. Lethal components, such as diphtheria toxin (DT) [113, 114] and *Pseudomonas* spp. exotoxin A (PE) [115, 116], are modified and attached to antibodies [117-122]. PE, a protein of 66 kD, consists of three domains: domain I is responsible for binding to cell surface receptor; domain II determines protein penetration into the cell cytoplasm, and domain III is responsible for ADP-ribosylation of eukaryotic elongation factor-2 (EF-2) [123]. Diphtheria toxin consists of two polypeptide chains linked by a disulfide bridge and has molecular mass of 62-63 kD [113, 114]. Its B-chain has a site for cell binding (domain I) and a site (domain II) responsible for transmembrane translocation of the A-chain (domain III) into cells. A-Chain is responsible for ADP-ribosylation of EF-2 [124-126]. DT and PE toxins translocating into cells cause ADP-ribosylation of EF-2, accompanied by its inactivation; this arrests protein synthesis and finally causes cell death [127, 128]. The hybrid protein Fv-p53, which consists of Fv-fragment of mAb 3E10 antibody and p53 protein, is toxic for cancer cells which became malignant due to mutation in the *p53* gene [129]. Immunoconjugates can contain components stimulating immune response. For example, recombinant antibodies linked to immunostimulator cytokines such as interleukin-2 (IL-2), interleukin-12 (IL-12), and granulocyte and macrophage colony stimulating factor (GM-CSF) have been constructed [130-132]. The combination of specific antibody targeting and immunostimulation by cytokines gives high cytokine concentration in the tumor microenvironment and, consequently, increased antitumor activity of antibodies and effective secondary antitumor immune response [132]. The recombinant protein scFvCD7:sTRAIL induced apoptosis in human tumor T-cells and exhibited low toxicity for normal blood and endothelial human cells. This scFvCD7:sTRAIL protein consists of TRAIL, the apoptosis-inducing ligand related to tumor necrosis factor, genetically fused with a single chain (sc) Fv-antibody fragment, which specifically binds to CD7 antigen on the surface of T-cells. Apoptotic signal induced by scFvCD7:sTRAIL was more potent than that of immunotoxin scFvCD7:ETA [133]. Death receptor ligands (CD95L, TNF, and TRAIL) are widely used as the antitumor agents due to their apoptosis inducing ability [134].

Development of recombinant antibodies (antibody fused to an effector molecule) is an alternative strategy to use of vectors providing transcription of therapeutic genes into target cells.

DEVELOPMENT OF VECTORS EXPRESSING SECRETED PROTEINS

The development of expression vectors for generating recombinant eukaryotic secreted fusion proteins exhibiting anticancer properties is a new trend in cancer therapy. Such proteins contain antibody to the specific tumor marker and effector molecule responsible for tumor regression like immunoconjugates described in the previous section. However, these proteins are synthesized in eukaryotic cells. For example, a chimeric construction containing IL-12 and antibody against HER2/neu has been developed. IL-12 is a heterodimer cytokine exhibiting antitumor and anti-metastatic effects. Cells transfected with this construction were able to secrete the chimeric protein of 320 kD. It binds to cells transfected with HER2/neu-antigen and thus preserves specificity typical for antibody against HER2/neu and cell expression IL-12 receptor. Analysis of T-cell proliferation and natural killer cytotoxic activity demonstrated that in the fused protein biological activity of IL-12 was comparable to that of murine IL-12. The hybrid protein also exhibited antitumor activity. Consequently, this recombinant protein effectively combines the therapeutic potential of IL-12 with selective binding of antibody to tumor cells; thus this protein may represent an alternative to therapeutic administration of IL-12 [135]. Granulocyte-macrophage colony stimulating factor (GM-CSF) was also used for tumor regression. The vector encoding secreted anti-Human HER2/neu IgG3-(GM-CSF) fused protein, containing humanized antibody against HER2/neu and murine cytokine GM-CSF has been constructed. Myeloid cells transfected with this vector secreted anti-Human HER2/neu IgG3-(GM-CSF) into the cultivation medium. The fused anti-Human HER2/neu IgG3-(GM-CSF) protein was able to bind HER2/neu-antigen and maintain growth of GM-CSF-dependent FDC-P1 murine myeloid cell line. It bound to the HER2/neu-expressing CT26-HER2/neu murine colon adenocarcinoma cells and activated J774.2 macrophage cell line. The chimeric protein anti-HER2/neu IgG3-(GM-CSF) provided GM-CSF-mediated stimulation of immunity at the tumor site. It was able to increase Th1- and Th2-immune response, B-cell immune response, and significantly reduced growth of CT26-HER2/neu tumor xenograft in mice [136].

Thus, the development of gene therapy employing secreted proteins began with design of chimeric constructs expressing hybrid proteins that contain therapeutic agents stimulating immune response. Vectors carrying genes that encoded cell death proteins were also functionally competent. For example, vector encoding secreted hybrid protein, which consisted of antibody against HER2, exotoxin A translocation domain, and caspase-3, exhibited binding to HER2-positive tumor cells, internalization into these cells, and selective killing of these cells [137]. Proapoptotic proteins are also promising agents for cancer therapy. There is a report on construction of an expression vector encoding single chain specific antibody against HER2, domain II of *Pseudomonas* sp. exotoxin A, and active caspase-6, which cleaves laminin A followed by

disintegration of the nucleus and induction of apoptosis. The secreted molecules of immunocaspase-6 recognized tumor cells overexpressing HER2 *in vitro* and induced apoptosis [138]. Serine protease granzyme B (GrB) triggers apoptosis through caspase-dependent and caspase-independent pathways [139, 140]. This explains high popularity of this enzyme in therapeutic agents. The constructed vector expressing secreted hybrid protein, which contained a single chain of the antibody against HER2, *Pseudomonas* exotoxin A translocating domain, and active GrB, was used for transfection of human Jurkat lymphoma cells. The secreted molecule of immunoGrB selectively recognized and killed tumor cells overexpressing HER2; this effect was observed both *in vitro* and in nude mice after intramuscular injection of a plasmid expressing immunoGrB. Subsequent studies *in vivo* revealed that intravenous injection of immunoGrB genetically modified lymphocytes suppressed HER2-overexpressing tumor cells and increased life span of tumor-bearing animals; this was achieved due to long-term secretion of immunoGrB molecules into blood and lymph. Thus, the chimeric immunoGrB molecule was specifically directed to HER2. Consequently, the constructed vector has therapeutic potential for reduction of HER2-positive tumors especially in the cases of inhibition of caspase-dependent apoptosis [141]. Chimeric constructions may employ ribonucleases as the toxic component. For example, human ribonuclease was used for construction of immunoconjugates against carcinoma cells overexpressing HER2/neu [142]. Barnase, *Bacillus amyloliquefaciens* ribonuclease [143], was used in constructs developed for expression in neuroblastoma (Neuro2) cells [144]. Stable Neuro2A cell clones expressing barnase under control of tetracycline controlled transactivator protein (tTA) exerted cell death only after tetracycline withdrawal, correlating with a 10-fold induction of barnase mRNA expression. Introduction of specific enolase promoter to this construct resulted in tissue-specific expression of barnase and neuronal cell death after tetracycline withdrawal both in cell culture and in transgenic mice [144]. Recently, a vector expressing a hybrid protein, anti-HER2/neu mini-antibody-barnase, which consists of scFv-fragment of HER2/neu antibody and barnase, has been constructed [145]. Expression of the fused protein anti-HER2/neu mini-antibody-barnase was under control of TRE (tetracycline responsive element), the minimal promoter in the Tet-Off gene expression system [10, 146]. The basal level of the suicidal gene barnase in this system was sensitive to inhibition by barstar, controlled by its own cytomegalovirus promoter. *B. amyloliquefaciens* barstar specifically binds barnase with formation of a highly stable complex, inhibiting barnase activity [146-149]. Overexpression of the fused protein anti-HER2/neu mini-antibody-barnase occurs in tetracycline-free cultivation medium, and transfected cells secrete the hybrid protein into the cultivation medium. Since HER2/neu is a ligand-internalizing receptor [150, 151], binding of the secreted hybrid protein to the cell surface receptor can be accompanied by its penetration into HER2/neu-positive cells, in which barnase would act as a ribonuclease.

Thus, besides targeted delivery of therapeutic anticancer agents using transcription activation of specific promoters or antibodies, a third mode of delivery is now actively developed. It is based on the use of vectors expressing secreted hybrid proteins containing antibody and protein therapeutic agent. The design of such vectors joins the method of gene therapy and therapy using antibody. Since anticancer gene therapy represents a new direction, the development of various modes of malignant tumor reduction is necessary and promising.

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