INTRODUCTION
The original concept of gene therapy is to treat and cure diseases caused by a known monogenic defect by introducing and expressing a normal copy of the mutated or deleted gene into the host cells. In this regard, gene therapy for cancer should be aimed at correcting gene alternations in cancer cells, that is, replacement of tumor suppressor genes and inactivation of oncogenes. However, cancer gene therapy has evolved in somewhat different directions. These include (i) transfer of suicide genes that convert inactive prodrugs into cytotoxic compounds, (ii) transfer of genes coding immuno-stimulators such as cytokines and chemokines to enhance anti-tumor immunity, (iii) transfer of genes coding anti-angiogenic factors to inhibit angiogenesis in solid tumors, (iv) transfer of drug resistant genes into normal hematopoietic stem cells to render them resistant to high-dose myelosuppressive chemotherapeutic agents. These strategies do not constitute “gene-replacement therapy” as defined above, and might instead be called “DNA therapeutics” for instance (1). Cancer gene therapy can be defined simply as “the transfer of nucleic acids into cancer or normal cells to eliminate or reduce tumor burden”.

Genes can be introduced into target cells \textit{ex vivo} and placed back into the host or directly into target cells \textit{in vivo}. Viral or non-viral vectors are used to facilitate the transfer of genes into target cells. This chapter discusses recent advances in gene therapy of the thyroid cancer field. Attention is focused on the therapeutic genes used.
STRATEGIES USED FOR THYROID CANCER GENE THERAPY

Silencing of oncogenes

According to the original concept of gene therapy, that is, “gene-replacement therapy”, we may speculate that correction of a mutated or an aberrantly overexpressed oncogene might reverse malignant phenotype. On the other hand, some may contend that since cancers generally arises as the culmination of a multiple process that involves a variety of somatic gene alternations (see Chapter 1 for more detail), it is impossible to correct all the genetic abnormalities, as neither to restore normal gene function in every cancer cells with currently available vectors.

Several mutations or overexpression of oncogenes have been identified in thyroid cancers. The former includes RAS mutations and RET gene rearrangements in follicular and papillary carcinomas, respectively (2), and the latter overexpression of c-myc and high mobility group I (Y) protein [HMG I (Y)] in some thyroid cancers with highly malignant phenotypes (3, 4). Theoretically, suppression of gene expression can possibly be achieved with antisense, ribozyme, intracellular single-chain antibodies or RNA interference. For instance, suppression by antisense method of expression of c-myc and HMG I (Y) protein is reported to induce growth inhibition and cell death, respectively, in thyroid cancer cell lines with overexpression of a respective gene (3, 4).

Replacement of tumor suppressor gene

Among numerous mutations in different tumor suppressor genes so far identified in distinct types of cancers, the gene for tumor suppressor p53 (5) is well known to be frequently mutated in anaplastic, not well-differentiated, thyroid carcinoma (6–8). These mutations are closely associated with de-differentiation of thyroid cancer, and therefore thought to be the late event in thyroid carcinogenesis.

One can expect that introduction of wild type (wt)-p53 gene into thyroid cancer cells defective in normal p53 might reverse malignant phenotype or induce re-differentiation. Indeed it has been reported that reintroduction of wt-p53 by stable transfection into p53-defective follicular cell-derived thyroid cancer cell lines and a medullary thyroid cancer (MTC) cell line led to cell cycle arrest and growth inhibition (presumably the cells expressing p53 at relatively low levels survived) (9–16). Re-expression of wt-p53 is accompanied by chemosensitization, radiosensitization and reappearance of the differentiated markers such as TPO, TSHR and PAX8 (9–11,14,15). Besides, of interest, despite in vitro cell growth inhibitory, not cell-killing, effect of wt-p53 in an anaplastic thyroid cancer cell line FRO, FRO cells stably expressing wt-p53 exhibits poor tumorigenicity in nude mice (16). Thus, tumors can not grow more than a few mm in a diameter. Tumors are found to be in an angiogenesis-restricted dormant state, that is, growth of FRO cells is counterbalanced with apoptotic cell death induced by anti-angiogenic effect of wt-p53. Wt-p53 appears to exert more complex anti-cancer actions than expected from in vitro data.

In contrast, however, high level expression of wt-p53 achieved with recombinant adenovirus clearly induces apoptotic cell death in vitro (17, 18). Furthermore, in in vivo experiments in nude mice, intratumoral injection of adenovirus expressing wt-p53
(1 x 10^9 pfu/tumor) into pre-established FRO tumors almost completely inhibited tumor growth and induced a small but significant tumor reduction when combined with doxorubicin (18). Of interest, it has recently been shown that a histone deacetylase inhibitor (depsipeptide) increases p53 transcriptional activity and thereby p21 expression, a downstream target of p53, and leads to enhancement of p53’s anti-tumor effect (19). Since anaplastic thyroid cancer is highly aggressive and refractory to conventional treatments, p53 gene therapy may be a promising new strategy for this type of cancer.

**Suicide gene/prodrug**

Genes whose products convert a relatively nontoxic prodrug into its toxic form are referred as “suicide genes” (20). Herpes simplex virus-thymidine kinase (HSV-TK), a most widely used suicide gene product, phosphorylates a prodrug ganciclovir (GCV) ~1000-fold more efficiently than mammalian TK. The resultant GCV monophosphate is further phosphorylated by the mammalian enzyme to GCV triphosphate, which inhibits DNA polymerase and is thus cytotoxic. *E. coli* Cytosine deaminase converts nontoxic 5-fluorocytosine to toxic 5-fluorouracil (5-FU) by deamination, which blocks thymidylate synthetase and mRNA transcription. Deoxycytidine kinase phosphorylates and activates a number of anti-neoplastic nucleotide analogues including cytosine arabinoside (Ara-C). *E. coli* Nitroreductase converts a prodrug CD1954 to its toxic form. The in vitro efficacy of all these combinations was confirmed in several thyroid cancer cell lines (21, 22). Nishihara et al. (21) have also demonstrated HSV-TK/GCV-mediated radiosensitization in thyroid cancer cells. Although it is impossible to transduce a therapeutic gene into every cancer cells in vivo with currently available vectors, non-transduced cells can be killed by neighboring transduced cells, a phenomenon called “bystander effect”. Phosphorylated GCV can be transferred from transduced cells to adjacent non-transduced cells through gap junctions and phagocytosis of apoptotic vesicles of dead cells by live tumor cells. Induction of active, local immune response against tumors may participate in in vivo bystander effect. In addition, 5-FU, phosphorylated Ara-C and toxic CB1954 can also be secreted and taken up by surrounding cells. In this regard, these latter three combinations seem to be more efficacious than HSV-TK/GCV (22). Nevertheless, most of thyroid cancer gene therapy has been performed with HSV-TK/GCV.

HSV-TK and GCV system exerts their cytotoxic effect on not only proliferating cells (including cancer cells) but also metabolically active, non-proliferating cells such as normal thyroid cells (23, 24). It is therefore necessary to target expression of HSV-TK to cancer cells. One of the methods for targeting is transcriptional control of therapeutic gene expression. Although no thyroid cancer-specific promoter has been identified, several tissue-specific promoters are available [thyroglobulin (Tg), calcitonin (CT), etc.]. The preliminary experiments suggesting the potential usefulness of Tg promoter for thyroid cancer gene therapy have been performed in vitro with normal, differentiated rat thyroid cell line FRTL5 by Zeiger et al. (25). Subsequently with the retrovirus vector and transformed rat FRTC cells, a model for differentiated thyroid cancer cells, Braiden et al. (26) have demonstrated the feasibility of Tg promoter and
HSV-TK/GCV system *in vitro* and *in vivo*. Zhang *et al.* (27, 28) have recently demonstrated not only the efficacy but also the safety of Tg promoter with adenovirus *in vivo*. Thus intravenous (i.v.) injection of adenovirus containing HSV-TK gene under the control of Tg promoter did not induce liver damage (the main target organ for i.v. adenovirus).

However, the activities of tissue-specific promoters are generally weaker than those of constitutive viral promoters [e.g., human cytomegalovirus (CMV) promoter]. To overcome this drawback, use of Cre-\textit{loxP} system is one option (29). In this study, two adenovirus vectors were constructed; one contained the expression cassette of Tg promoter and Cre recombinase gene, and the other of CMV promoter and HSV-TK gene which were interrupted with two \textit{loxP} sequences flanking the neomycin-resistance gene. When these two adenoviruses were co-infected into the cells in which Tg promoter is active, Cre was expressed from Tg promoter in the first vector, and excised the neomycin-resistance sequence and placed HSV-TK gene under the control of the CMV promoter in the second vector, which exhibited the enhanced therapeutic effect as compared with the combination of Tg promoter and HSV-TK gene. However, it should be noted here that the need of double infection might curtail the therapeutic efficacy when multiplicity of infection (MOI) is low or tumor cells are resistant to adenovirus infection. In addition, Takeda *et al.* (30) have reported the higher efficacy of a tandemly repeated Tg promoters.

Another problem for use of tissue-specific promoters is loss of tissue-specific promoter activities in poorly differentiated and anaplastic thyroid cancer, making Tg promoter useless for treatment of these types of thyroid cancer. Two studies suggest the potential usefulness of thyroid-related transcription factors to re-activate Tg promoter in thyroid cancer cell lines with no Tg expression, but data are somewhat different (30, 31). Chun *et al.* (31) have shown enhancement of Tg promoter by co-transfection of thyroid transcription factor-1 (TTF-1) and PAX-8, while TTF-1 alone was sufficient in studies by Shimura *et al.* (32). The different cell lines used (ARO and WRO cells versus FRT and BHP15-3 cells) may explain these different results. Further studies will be necessary to clarify this controversy, a very important point considering clinical trial in the future.

Furthermore, Kitazono *et al.* (33, 34) have found that histone deacetylase inhibitors (depsipeptide and sodium butyrate) enhanced activity of Tg promoter or Tg enhancer/promoter and this effect was further augmented by a cAMP analogue in thyroid cancer cell lines with no Tg expression.

Similar studies with HSV-TK/GCV and a tissue-specific promoter (CT promoter) have also been performed in MTC with a rat MTC cell line, 6–23 (clone 6), and a human MTC cell line, TT, *in vitro* and with 6–23 cells *in vivo* (35, 36).

Finally, Soler *et al.* (37) have used nitric oxide synthase II (NOS II) gene as a suicide gene for treatment of MTC. NOS II produces NO which is the main mediator of the tumoricidal action of activated macrophage. Despite an extremely low gene transfer efficiency (~1 %), injection of the naked plasmid containing CMV promoter and NOS II cDNA into orthotopically established MTC tumors led to tumor growth inhibition,
suggesting marked bystander effect probably due to NO diffusion. Thus NOS II gene can also be used as a suicide gene in cancer gene therapy.

Enhancement of tumor immunity

Cancer cells can be recognized as a foreign by host immune system. However, this anti-tumor immune response is usually not strong enough to eradicate tumors. The mechanisms of insufficient anti-tumor immunity include loss of expression of major histocompatibility complex (MHC) antigens and/or co-stimulatory molecules on cancer cells, and secretion of immuno-suppressive cytokine(s) (e.g., TGF-β) from cancer cells. Systemic administration of cytokine(s) can be used to enhance immune response to tumor antigens, but is always accompanied by undesirable side effects. To overcome these problems, cDNAs coding cytokines, MHC or co-stimulatory molecules have been introduced into tumor cells to make tumor cells more immuno-genic and reduce the toxicity. Two approaches are usually employed; immunization with transduced (and irradiated) autologous tumor cells or in situ gene delivery into an established tumor mass.

To my knowledge, the first article describing immune-gene therapy against thyroid cancer is one by Lausson et al. (38). They showed that rat MTC cells stably expressing interleukin-2 (IL-2) injected subcutaneously or orthotopically were completely rejected in syngeneic rats. This anti-tumor effect appeared to involve the recruitment of CD8+ T lymphocytes. Subsequently, DeGroot and his colleagues performed extensive studies on immuno-gene therapy for MTC with cytokines in rat and mouse MTC models. Tumorigenicity of mouse MTC cells infected with adenovirus harboring IL-2 gene (under the control of CMV promoter) was shown very poor in syngeneic immuno-competent mice (39). Established long lasting immunity was demonstrated by re-challenge with parental MTC cells in protected mice. Cell-mediated cytotoxic assays showed that both cytotoxic T lymphocytes and NK cells play a role. Loss of tumorigenesis of MTC cells infected with adenovirus expressing IL-2 in severe combined immune deficiency mice also indicates involvement of NK cells in this anti-tumor immunity (39). In in vivo situations where adenovirus expressing IL-2 (1 × 10^9 pfu in mice and 2 × 10^9 pfu in rat) was injected into pre-established MTC tumors, 70% and 43%, respectively, of the small tumors (<30 mm^3 in mice and <100 mm^3 in rat) were eradicated, but all the large tumors (>30 mm^3 in mice and >100 mm^3 in rat) showed stabilization in size (40, 41). They have also addressed the safety issue of intratumoral inoculation of adenovirus harboring IL-2 gene under the control of constitutive CMV promoter (41). Despite detection of dissemination of inoculated adenovirus from tumor to liver, no liver dysfunction was observed except mild pathological change (lymphocyte infiltration) even when constitutive viral promoter was used to drive IL-2 expression, suggesting that direct injection of adenovirus expressing IL-2 (and presumably other cytokines) can be safe. Their recent studies have demonstrated that IL-12 appears to be more efficacious than IL-2 (42). In a rat MTC model, the cure rate was 100% in smaller tumors (<100 mm^3) injected with 1 × 10^9 pfu adenovirus expressing IL-12, versus 43% complete eradication with 2 × 10^9 pfu adenovirus
expressing IL-2 in the aforementioned report (41). Seventy-eight % of large tumors (>100 mm³) was also eradicated (versus 0% in case of IL-2). Furthermore, they showed intravenous injection of adenovirus coding IL-12 was safe when IL-12 gene expression was confined to MTC tumors (and thyroid parafollicular C cells) by using the modified CT promoter comprised of two tandemly arranged tissue-specific enhancer elements and a minimal proximal CT promoter (43). More recently the efficacy of adenovirus expressing IL-12 has also been shown in thyroid follicular cancer (44).

In addition, enhanced effect of the combined HSV-TK/GCV and IL-2 has also been demonstrated by three groups (45–47). For example, in studies by DeGroot’s group (46), the complete eradication of pre-established MTC tumors was induced in 63% of mice treated with adenovirus expressing HSV-TK and IL-2, 38% with adenovirus expressing IL-2 and 12% with adenovirus expressing HSV-TK (all pfu).

Finally, although no thyroid tumor rejection antigens have yet been identified, the possibility of preprocalcitonin ((PPCT) as a tumor rejection antigen in MTC has been investigated by a means of DNA immunization (48). Co-delivery of PPCT and granulocyte-macrophage colony-stimulating factor genes induced cellular and humoral immune responses against PPCT, suggesting a potential of DNA immunization as a novel immunotherapeutic treatment for MTC.

**Selectively replicative virus (Oncolytic virus)**

As shown above, recombinant adenovirus is being widely used as a vehicle for gene delivery in cancer gene therapy. In vivo therapeutic efficacy of non-replicative adenovirus is however limited mainly because of its low infectivity and poor gene delivery to a solid tumor. Use of replicative adenovirus is thus a potential candidate to overcome this issue (49). ONYX-015 is such an adenovirus with a deletion in E1B 55 kD gene and reportedly replicates selectively in the cells defective in p53 gene (50), although this p53 mutation-selective replication has been disputed (49). Intratumorally (or i.v.) injected ONYX-015 first infects to a small fraction of tumor cells, in which virus replicates and induces cell death (cytopathic effect), and then virus progeny released infects to surrounding tumor cells. Portella et al. (51) have recently demonstrated anti-tumor effect and chemosensitivity of ONYX-015 in several thyroid cancer cell lines defective in wt-p53. They have also addressed the safety of this virus using a rat normal thyroid cell line PC C13. However, one should be cautious for these data, because human adenovirus does not usually replicate well in non-human (eg., rodent) cells. Indeed we have previously found that selectively replicative adenovirus can replicate and produce progeny in normal human thyroid cells in culture. Further, there is no difference in viral replication between anaplastic thyroid cancer cell line FRO and FRO cells stably expressing wt-p53, suggesting that adenovirus replication appears independent from p53 status (our unpublished data). These data does not exclude the use of replicative adenovirus for thyroid cancer treatment, rather indicate that this type of oncolytic virus can be used for both differentiated and anaplastic thyroid cancers. In this case, there is a need to strictly control virus replication in order to avoid undesired viral spread. For example, we have used Cre-loxP system and p53-responsive promoter to control E1A protein expression, which is essential for adenovirus replication (52). This may be a
promising means to restrict virus replication to anaplastic thyroid cancer. Tg promoter can also be used to express E1A proteins in differentiated thyroid cancer (53).

However, it is usually impossible to completely eradicate tumors with replicative adenovirus alone. Therefore, multimodality treatment with other antitumor agents might be necessary. Indeed, replicative adenovirus has also been reported to work synergistically with chemotherapy. Also replicative adenovirus can be armed with a therapeutic gene such as a suicide or a cytokine gene (54).

**Antiangiogenic factors**

It is well known that solid tumors can not grow more than a few mm³ without oxygen and nutrient supplied from blood (55), suggesting that new vessel formation (called angiogenesis) is a prerequisite for solid tumor growth. Therefore, inhibition of angiogenesis might be a promising strategy for cancer treatment. Numerous antiangiogenic factors have so far been isolated such as angiostatin, endostatin, etc. Since these agents act basically on normal vascular endothelial cells, resistance to these agents can not be easily induced. To my knowledge, only one study describes the effect of an antiangiogenic factor gene on thyroid cancer; thrombomodulin-1 inhibits angiogenesis and growth of FRO tumors (16). As mentioned above, this article also demonstrates the ability of wt-p53 to induce anti-angiogenesis-mediated dormancy.

**Iodide transporter**

Active influx and efflux of iodide in the thyroid gland are mediated by sodium iodide symporter (NIS) and chloride-iodide transporter (Pendrin), respectively (56, 57). Failure of iodide concentration in some differentiated and most anaplastic thyroid cancers are generally due to decreased or loss of NIS expression. Therefore, targeted expression of NIS gene in thyroid cancers with no or little NIS expression (and also non-thyroid cancers) would offer the possibility of radioiodide therapy (58, 59). Shimura et al. (60) have first shown the significant iodide concentration in transformed rat thyroid cancer cells genetically engineered to express NIS. However, no or weak efficacy was demonstrated in in vivo studies (61, 62) because of rapid efflux of iodide. Co-expression of thyroid peroxidase has recently been demonstrated to augment iodide retention in the cells by iodide organification (63). In addition, a histone deacetylase inhibitor (Trichostatin A) has been reported to increase NIS expression and decrease Pendrin expression (64). In contrast, expression of endogenous NIS in breast cancer can be diagnostically and therapeutically useful (65). Further studies will be needed to investigate the possibility of NIS gene for cancer gene therapy.

**CONCLUSIONS**

I here summarized the recent articles regarding gene therapy for thyroid cancer. Although there have been tremendous progresses in this field in the last decade, there is unfortunately no published report on clinical trial of gene therapy for thyroid cancer [except one patient treated with ONYX-015 (66)]. Patients with thyroid cancer, particularly those with anaplastic and medullary cancers, will hopefully benefit from gene therapy approach in the near future.
REFERENCES

21. Gene therapy for thyroid cancer