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In vivo delivery technique of nucleic acid compounds using atelocollagen: Its use in cancer therapeutics targeted at the heparin-binding growth factor midkine

Review Article

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Abbreviations: alanine aminotransferase, (ALT); aspartate aminotransferase, (AST); blood urea nitrogen, (BUN); drug delivery system, (DDS); ethoxylated polyethylenimine, (EPEI); fetal bovine serum, (FBS); fluorescein isothiocyanate, (FITC); gene activated matrix, (GAM); inverted-thymidine-modified antisense DNA, (Inverted T AS); matrix-assisted laser desorption/ionization time of flight, (MALDI-TOF); midkine, (MK); morpholino antisense oligomers, (Morpho/ASs); phosphodiester, (PO); phosphorothioate, (PS); poly(lactide-co-glycolide), (PLCG); poly[alpha-(4-aminobutyl)-L-glycolic acid], (PAGA); polyethylene vinyl co-acetate, (EVAc); RNA interference, (RNAi); small interfering RNA, (siRNA); vascular endothelial growth factor, (VEGF)

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Summary

The largest obstacle in developing a therapeutic based on nucleic acid compounds, including antisense DNA, ribozyme and small interfering RNA, lies in the necessity of achieving effective transfection into the target organ and tissue. In this review, we introduce a technique to deliver nucleic acid compounds to tissue *in vivo* using atelocollagen. Atelocollagen, a pepsin-digested product of type I collagen derived from the dermis of cattle, is liquidified at low temperature and gels at 37ÆC. These unique properties of atelocollagen provide a nuclease-resistant *in vivo* environment and tissue maintenance of nucleic acid-based injected therapeutics, thus ensuring maximal treatment efficacy. In this review we introduce antisense DNA, morpholino antisense oligomer and siRNA targeting midkine a heparin-binding growth factor that is overexpressed in various tumors of humans and discuss their application in cancer therapy. All three nucleic acid compounds were transfected into xenografted tumor cells *in vivo* at high efficiency when they were mixed with atelocollagen. We proved that siRNA mixed with atelocollagen stayed in the tumor for at least eight days and was maintained intact. This review will be practically useful for developing a nucleic acid-based therapeutic against various diseases, including cancers.

I. Introduction

Recently, advances in cancer cell biology has not only elucidated the genetic alterations and mechanisms underlying abnormal replication specific to cancer cells, but have also contributed to the development and clinical application of new drugs with fewer adverse effect that are targeted at the altered and/or upregulated molecules in cancer cells. These molecular targeting drugs have the advantage of exerting their effect specifically on the cancer cells expressing the target molecules.

Midkine (MK), a heparin-binding growth factor, is upregulated in most cancer tissue and succeeded in its cDNA cloning (Kadomatsu et al, 1988; Tsutsui et al, 1993; Aridome et al, 1995). In this paper we introduce a new type of anticancer therapy with few adverse effects that is targeted at MK. To suppress the expression of the MK gene and protein, we selected antisense and RNA interference (RNAi) methods. We first constructed phosphorothioate (PS)-modified antisense DNA, morpholino antisense oligomers (Morpho/ASs) and small interfering RNA (siRNA) *in vitro* to suppress MK

expression and then carried out treatment experiments using a subcutaneous tumor model in nude mice. We used atelocollagen, a biomaterial, for the delivery of antisense DNA into the tumor.

II. Atelocollagen

Gene introduction techniques aim to deliver gene expression vectors and antisense DNA to cells in the whole body or, specifically, to the targeted disease site at the optimal concentration and timing. For maximal therapeutic effects a biomaterial (biophilic substance) is most often used as a carrier (Ochiya et al, 2001). Recently, novel bio-introduction techniques using biomaterials combined with nucleic acid compounds, which are required for the conventional drug delivery system (DDS) and successful gene therapy, have been developed. The following biomaterials have been reported to be effective for gene introduction: polyethylene vinyl co-acetate (EVAc, Luo et al, 1999), poly(lactide-co-glycolide) (PLCG, Shea et al, 1999), gene activated matrix (GAM, Bonadio et al, 1999), poly[alpha-(4-aminobutyl)-L-glycolic acid] (PAGA, Lim et al, 2000), imidazole-containing polymer (Pack et al, 2000), alginate microsphere (Mittal et al, 2001), chitosan (Roy et al, 1999), gelatin (Truong-Le et al, 1999), PLA-DX-PEG (Saito et al, 2001) and atelocollagen (Ochiya et al, 1999).

We here introduce a technique to deliver nucleic acid compounds to tissue *in vivo* using atelocollagen. The collagen molecule forms a helix consisting of three polypeptide chains and exhibits a stick-like structure, 300 nm in length and 1.5 nm in diameter. This molecule has telopeptides at both ends, which account for most of the antigenicity of collagen. It becomes atelocollagen and loses most of its antigenicity after removal of the telopeptides by pepsin digestion (Fujioka et al, 1995). The structure of atelocollagen is shown in **Figure 1**. Atelocollagen is liquidified at low temperature (10°C or lower) and gels at 37°C. Therefore, mixing nucleic acid compounds with atelocollagen at low temperature before embedding or inoculating them into animals allow nucleic acid compounds to stay at the inoculation site (**Figure 2**). Controlled release in the body of embedded nucleic acid compounds is achieved by digestion with collagenase.

III. Midkine: Our target gene for cancer therapy

The heparin-binding growth factor MK is a basic, cysteine-rich 13-kDa polypeptide having about 50% sequence identity with pleiotrophin/heparin-binding growth-associated molecule (Kadomatsu et al, 1988; Rauvala, 1989; Tomomura et al, 1990; Muramatsu, 2002; Kadomatsu and Muramatsu, 2004). MK is broadly distributed in vertebrates from zebra fish to humans. To date, the molecular structure of MK has been determined in humans, rats, mice, rabbits, cows, chickens and Xenopus. Human and mouse MK share 87% sequence identity (Tsutsui et al, 1991). MK was found to be the product of a retinoic acid-responsive gene discovered by screening for induced genes during the differentiation of embryonal carcinoma cells (Kadomatsu et al, 1989). In fact, the 5'-regulatory region of both human and mouse MK genes contains one retinoic acid-responsive element (Matsubara et al, 1994). Chicken MK is also called retinoic acid-inducible heparin-binding protein (Vigny et al, 1989; Raulais et al, 1991).

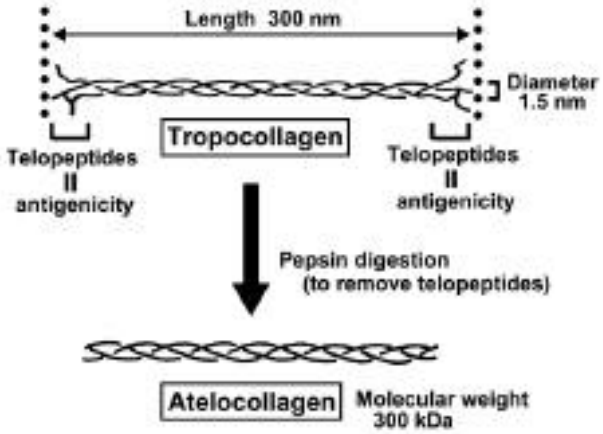

Figure 1. Schematic representation of atelocollagen molecule.

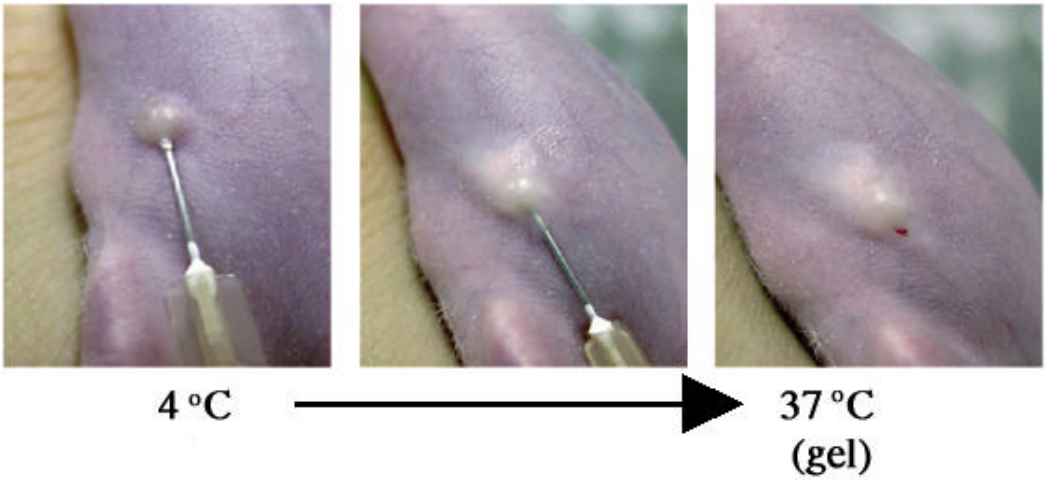

Figure 2. Injection of atelocollagen/nucleic acid compound complex in artificial subcutanerous tumors.

Despite the limited expression of MK in adult normal tissue, MK expression is increased in a number of malignant tumors compared to the adjacent non-cancerous tissue, including esophageal, stomach, colon, hepatocellular, breast, thyroid, lung, prostate and urinary bladder carcinomas, Hodgkin's disease, cholangiocarcinoma, Wilms' tumor, neuroblastoma, glioblastoma and other brain tumors. (Garver et al, 1993, 1994; Tsutsui et al, 1993; Aridome et al, 1995; Nakagawara et al, 1995; O'Brien et al, 1996; Mishima et al, 1997; Kato S et al, 1999; Koide et al, 1999; Konishi 1999; Kato H et al, 2000; Kato M et al, 2000a, 2000b) The frequency of overexpression depends on the tumor type. Thus, in gastrointestinal carcinomas MK is overexpressed in about 80% of cases and in prostate carcinoma MK is already detectable at the early stage (Konishi et al, 1999). In colon carcinogenesis, MK expression increases at the adenoma stage and the intensity increases during tumor progression (Ye et al, 1999).

Serum MK concentrations were elevated in a variety of cancer patients (Ikematsu et al, 2000) and we demonstrated that serum MK elevation correlated with factors indicating a poor prognosis (e. g. amplification of MYCN and downregulation of Trk A) in patients with neuroblastoma (Ikematsu et al, 2003). These findings suggest that MK accelerates the growth and development of a variety of cancers and is an excellent target molecule for cancer therapy. On the basis of this research background, we started developing antisense DNA and siRNA that would inhibit the MK gene and protein expression and carried out experiments to apply these compounds in cancer treatment.

IV. PS-modified antisense DNA against mouse MK

PS-modified antisense DNA was constructed to inhibit the expression of the mouse MK gene and protein (Takei et al, 2001). The secondary structure of the mouse MK mRNA was analyzed and four kinds of PS-modified antisense DNA (18-mer) were synthesized that were targeted at the loops, where base pairs are not formed. With a cationic liposome reagent (Lipofectamine-Plus, Invitrogen), the synthesized antisense DNA (final concentration, 5 μM) was transfected into mouse rectal carcinoma (CMT-93) cells, which abundantly secrete MK into the medium. Then the supernatant was subjected to Western blotting and the molecule of antisense DNA (AS) that inhibited MK production in CMT-93 cells was identified. AS treatment decreased MK production to about one-tenth of that in the cells without AS treatment. The control oligo DNAs, the sense sequence (SEN) and the reverse sequence (REV) caused no change in MK production.

AS treatment inhibited remarkably the proliferation of CMT-93 cells on day 3 or later. In contrast, the treatment with SEN hardly inhibited cell proliferation. Addition of chemically synthesized MK (5 ng/ml) to the medium of AS-treated cells restored cell proliferation to the level of that in SEN-treated cells. AS-treated CMT-93 cells showed a decreased colony formation in soft agar. Addition of MK to the soft agar restored colony formation to AS-treated cells. Taken together, these results indicated that MK played a critical role in the anchorage-dependent and -independent proliferation of CMT-93 cells.

Inoculation of AS-transfected CMT-93 cells into nude mice showed remarkable inhibition of tumorigenesis compared to non-AS-transfected tumor cells. Seven days after inoculation, an additional AS injection further inhibited the growth of the tumor in a concentration-dependent fashion.

Direct injection of the mixture of atelocollagen and AS into the CMT-93 tumor pre-grown in nude mice caused inhibition of the tumor growth. AS injection every two weeks caused increasing inhibition of tumor growth. The weight of the extracted tumor 41 days after AS treatment was significantly lower than that of an untreated tumor. In addition, the amounts of MK in the tumor were decreased in the AS-treated group on days 10, 17 and 24. Atelocollagen itself did not inhibit the tumor growth *in vivo*.

In the AS-treated tumor, a decrease in microvascular density and the number of 5-bromodeoxyuridine-positive cells was further examined; it was shown that AS treatment suppressed neovascularization and cell proliferation in the tumor. Taken together, these results suggested that MK accelerated intratumor neovascularization.

In these experiments, atelocollagen was employed for the delivery of AS into the tumor. On the other hand, direct AS injection into the tumor without mixing it with atelocollagen showed only slight antitumor effect.

V. Inverted thymidine-modified antisense DNA against mouse MK

Rapid degradation of the 'natural' phosphodiester (PO) backbone oligonucleotides by nucleases (Sands et al, 1994; Agrawal et al, 1995) necessitated chemical modification of the PO backbone (**Figure 3**). Chemical modifications, such as those seen in methylphosphonate (Smith et al, 1986; Sarin et al, 1988), PS (Matsukura et al, 1987; Agrawal et al, 1989) and phosphoramidate (Agrawal et al, 1988) oligonucleotides, have been introduced to make the oligonucleotides stable to degradative enzymes in serum (Sproat, 1988). Among these chemically modified compounds, PS-modified oligonucleotides are most frequently used because of their ease of manufacture, low cost and resistance to nucleases. However, PS-modified oligonucleotides have been shown to have toxic side effects in both cells in culture and in animals (Galbraith et al, 1994; Sarmiento et al, 1994; Henry et al, 1997; Monteith and Levin, 1999). The toxicity of PS-modified oligonucleotides derives from the replacement of oxygen atoms in the PO bond with sulfur atoms for stabilization against nucleases. Based on these researches, we have tried to develop novel oligonucleotides that are more resistant to degradation by nucleases and can knock down the target gene expression.

Focusing on the finding that DNA enzymes modified with inverted thymidine at the 3'-terminus is resistant to serum nucleases (Santiago et al, 1999; Sioud and Leirdal,

2000), we succeeded in chemically synthesizing inverted-thymidine-modified antisense DNA (Inverted T AS), which is single strand antisense DNA modified with inverted thymidine at the 5'- and 3'- termini (Takei et al, 2002). The structure is shown in **Figure 4**. Inverted T AS exhibits a structure in which inverted thymidine groups are bound to both ends of the core nucleotide sequence (18-mer), which can bind the target gene, mouse MK mRNA. CMT-93 cells overexpressing the target gene, mouse MK, were treated with PS-modified antisense DNA or Inverted T AS and both inhibited MK production to a similar degree. Modification with inverted thymidine groups did not cause a loss of antisense DNA functions.

The Inverted T AS, unlike PS-modified antisense DNA, showed little cytotoxicity. The half-life of the Inverted T AS in 5% FBS was as long as 110 hours (examined by PAGE). In contrast, the half-life of PS-modified antisense DNA was 10 hours and that of a DNA strand (20-mer) modified with regular thymidine residues at both ends, instead of inverted thymidine residues, was five hours. Taken together, terminal modification of a DNA strand (20-mer) with inverted thymidine residues was proven to be markedly resistant to serum nucleases. Furthermore, it became evident that short single strand

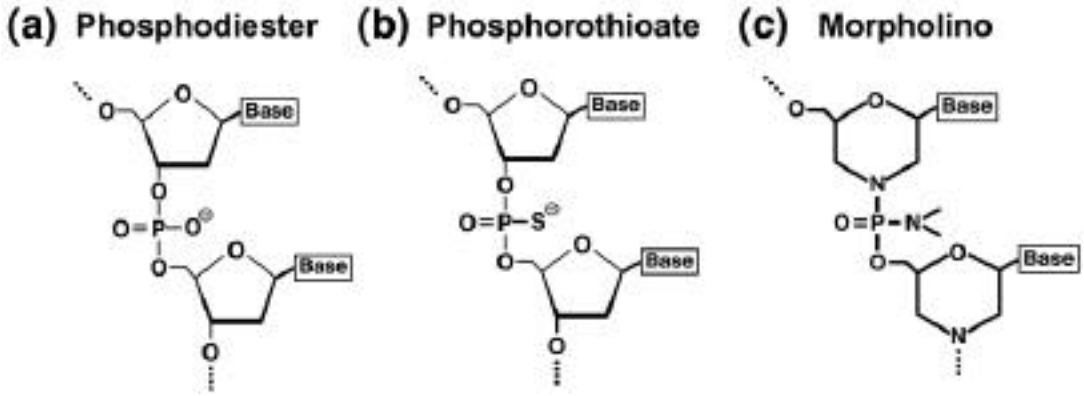

Figure 3. Chemical structures of (**a**) a phosphodiester oligomer, (**b**) a phosphorothioate oligomer and (**c**) a morpholino oligomer.

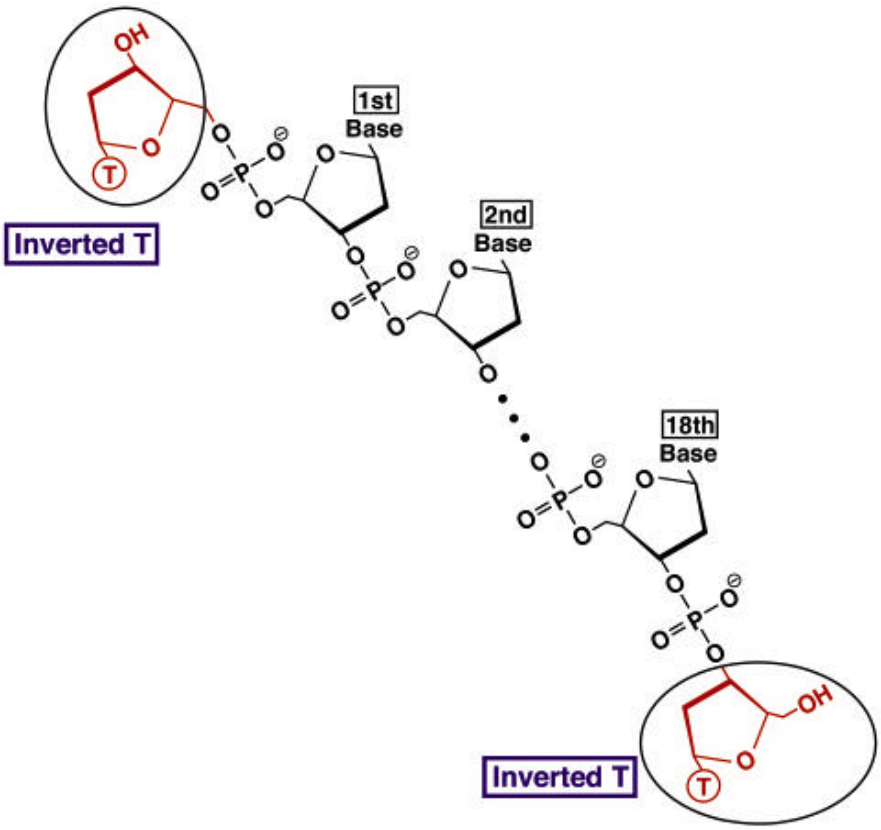

Figure 4. Chemical structure of an oligodeoxynucleotide modified with inverted thymidine at the 5'- and 3'-termini.

DNA (about 20-mer) was degraded mainly by serum exonucleases, but hardly by endonucleases.

Inverted T AS, targeted at the mouse MK gene, inhibited the proliferation of mouse rectal carcinoma cells, CMT-93, *in vitro* and injection of Inverted T AS mixed with atelocollagen into the CMT-93 tumor in nude mice remarkably inhibited the growth of the tumor (Takei et al, 2002). The antitumor effect exceeded that by PS-modified antisense DNA mixed with atelocollagen. We compared the efficacy of atelocollagen and cationic liposomes (Lipofectamine-plus, Invitrogen) in delivering antisense DNA into the CMT-93 tumor. Although the group using cationic liposomes as a carrier exhibited some antitumor effect, the group using atelocollagen showed a stronger antitumor effect. This result indicated that atelocollagen was by far superior to cationic liposome as a carrier for gene transfection *in vivo.* We also obtained evidence that antisense DNA was transfected to the inside of the cells with atelocollagen *in vivo* (Takei et al, unpublished results).

VI. Morpholino antisense oligomer against human MK

A morpholino antisense oligomer has overcome the problems (for example, specificity, stability and difficulty in determining effective nucleotide sequences) associated with PS-modified antisense DNA (Stein et al, 1997; Summerton and Weller, 1997; Summerton et al, 1997). It is a so-called third generation antisense backbone that exhibits virtually no cytotoxicity. Morpho/AS compounds are completely resistant to nucleases and superior in heat stability. Autoclave sterilization is possible.

The Tm value for the binding between a Morpho/AS and an RNA strand is higher than that between a natural DNA strand and an RNA strand, which allows stable binding (Summerton and Weller, 1997). Effective nucleotide sequences can be easily designed because a Morpho/AS has high affinity to the target mRNA and binds the target sequence independent of the secondary structure of the target mRNA (Summerton and Weller, 1997). Morpho/ASs exhibit high water solubility (230 mg dissolves in 1 ml of distilled water) and are easily dispensed. Since Morpho/ASs do not show nonspecific binding to proteins and high specificity can be maintained, they show the knockdown effect on the target gene at nanomolar concentrations. Focusing on a variety of advantages of Morpho/ASs, we constructed the system to knock down the expression of human MK.

We successfully constructed a Morpho/AS (25-mer) that suppressed human MK expression (Takei et al, 2005). The effective nucleotide sequence included the initiation codon ATG. The synthesized Morpho/AS (Gene Tools, U.S.; purity, 95% or higher by mass spectrometry) was transfected into human prostate carcinoma (PC-3) cells, which secreted MK into the culture medium, under serum-deprived conditions with a weakly basic gene transfection reagent (Ethoxylated polyethylenimine, EPEI; Gene Tools. Final concentration of Morpho/AS, 1 μM). After transfection (24 hours later), the supernatant was subjected to Western blotting, which showed that the Morpho/AS caused a decrease in the MK production in PC-3 cells to about one-tenth of that in the untreated cells. Control morpholino oligomers, the SEN and the REV, hardly changed MK production. The Morpho/AS also inhibited MK production and expression in human colon carcinoma SW620 cells. These inhibitory effects on MK expression by the Morpho/AS were dose-dependent.

With the effective nucleotide sequence of the Morpho/AS unchanged, antisense DNA with the phosphorothioate structure was constructed and transfected into PC-3 cells in a similar manner. Interestingly, when the morpholino backbone was replaced with the phosphorothioate backbone, the inhibitory effect on the expression of the target gene MK was completely eliminated. The reason for this difference is that the Morpho/AS and PS-modified antisense DNAs exhibit inhibitory effects on gene expression via different mechanisms. Thus, the Morpho/AS inhibits gene expression via the RNase-H independent mechanism, while PS-modified antisense DNA exerts its effect via the RNase-H-dependent (competent) mechanism (Summerton, 1999).

The Morpho/AS, which could suppress human MK expression, was transfected into PC-3 cells and the growth was inhibited. By contrast, the Morpho/SEN group showed little suppression of cell growth. In addition, the PC-3 cells, in which MK was knocked down by the Morpho/AS, formed significantly smaller colonies. Taken together, it can be concluded that MK plays an important role in both anchorage-dependent and -independent growth of PC-3 cells.

Injection of the Morpho/AS-atelocollagen complex into the transplanted PC-3 tumor three times at 14-day interval successfully inhibited the growth of the tumor. On day 41 after treatment, the tumor weight was significantly lower in the Morpho/AS group than in the control group.

The Morpho/AS did not show cytotoxicity *in vitro* at concentrations up to 200 μM. On the other hand, PS-modified antisense DNA showed cytotoxicity even at 50 μM. PS-modified antisense DNA at 50 nmol/mouse significantly increased BUN/creatinine and AST/ALT concentrations, indicating impaired renal and liver functions. The Morpho/AS was completely resistant to serum nucleases. For example, MALDI-TOF mass spectrometry demonstrated that the Morpho/AS, which was mixed with 5% FBS for 7 days and digested with proteinase K, showed the same profile as the Morpho/AS without such treatment. The Morpho/AS also exhibited complete resistance to nuclease S1 and nuclease P1.

VII. siRNA against human MK

Since the report by Elbashir et al, 2001, the knockdown method using RNAi has been established as the standard method to inhibit mammalian gene expression. The high sequence-specificity of RNAi has generated high expectations for its application to gene therapy for diseases such as cancer, AIDS and genetic disorders that are difficult to treat. We have already successfully developed the RNAi system to inhibit human MK expression *in vitro* (Takei et al, unpublished data).

Currently, cancer therapy studies using atelocollagen as an siRNA carrier are ongoing.

In the studies using siRNA for cancer treatment, we have constructed siRNA to knock down human vascular endothelial growth factor (VEGF) and have succeeded in delivering VEGF siRNA *in vivo* using atelocollagen (Takei et al, 2004). We have succeeded in knocking down VEGF expression both *in vitro* and *in vivo*. Injection of the mixture of VEGF siRNA and atelocollagen (50 μl complex/tumor) into the PC-3 xenografted tumor significantly inhibited tumor growth. The tumor treated with siRNA showed a decrease in intratumor microvascular density, which was dependent on the decrease of intratumor VEGF concentration caused by the siRNA. Atelocollagen contributed to the delivery of siRNAs into the tumor in two ways. (1) Increase of intratumor stability of the injected siRNA; (2) Efficient transfection of the injected siRNA into the tumor. We proved these *in vivo* effects of atelocollagen by labeling the VEGF siRNA with FITC and ^{32}P (Takei et al, 2004).

VIII. Conclusions

Atelocollagen is a markedly useful biomaterial for the *in vivo* delivery of nucleic acid compounds such as antisense DNA, Morpho/AS and siRNA. We proved that antisense DNA, the Morpho/AS and siRNA were all incorporated into tumor cells *in vivo* at high efficiency when they were mixed with atelocollagen. Antisense nucleic acid compounds, which could inhibit the heparin-binding growth factor midkine, inhibited the growth of cancer cells in nude mice. Atelocollagen promotes efficient transfection of antisense oligo DNA in cell culture systems *in vitro* (Honma et al, 2001) and is superior as a transfection reagent to cationic liposomes both *in vitro* and *in vivo*. Atelocollagen also protects the injected siRNA from nucleases *in vitro* (Minakuchi et al, 2004) as well as *in vivo* (Takei et al, 2004). We would like to study the effects of systemic delivery of antisense nucleic acid compounds in the future.

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