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Antisense oligodesoxynucleotide strategies in renal and cardiovascular disease

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Antisense oligodesoxynucleotide strategies in renal and cardiovascular disease. Antisense oligodesoxynucleoties (ODN) provide a novel strategy to inhibit RNA transcription and thereby the synthesis of the gene product. Because antisense ODN hybridize with the mRNA strand, they are highly specific. Their backbone structure has been modified to phosphorothioates or phosphoamidates so that they can better withstand degradation after delivery. We have shown that antisense ODN are a useful research tool to elucidate intracellular processes. The example we provide involves the inhibition of PKC signaling. Furthermore, we have shown the potential clinical utility of antisense treatment. We successfully inhibited the expression of the surface adhesion molecule ICAM-1 with antisense ODN in a model of reperfusion injury. This model is highly applicable to the problem of delayed graft function in humans. However, "getting there" is a major problem and clearly less than half the fun. Cationic substances such as lipofectin have worked sufficiently well in the experimental setting. Viral gene transfer offers a possibility; however, viruses produce an additional series of problems. Liposomes may not provide sufficient transfer efficiency. Coating liposomes with viral fusion proteins may offer an ideal way with which to deliver the goods into the cytoplasm of the target cell.

The use of antisense oligodeoxynucleotides (ODN) for the blockade of gene expression was introduced in 1978 by Zamecnik and Stephenson [1]. Due to the specificity of Watson-Crick base-pair hybridization, antisense ODN have been used extensively in attempts to inhibit expression of distinct genes both *in vitro* and *in vivo*. Figure 1 outlines a schematic view of how antisense ODN probably function to inhibit RNA uptake to ribosomes and thus, protein transcription. Although their precise mechanism of action has not been clarified, antisense ODN offer considerable promise as novel molecular therapeutic agents against diseases including AIDS, cancer, and inflammatory disorders. Furthermore, antisense ODN have been used in renal and cardiovascular medicine to unravel pathophysiological mechanisms, and experimentally as therapeutic agents [2]. We will discuss several aspects of our experience using antisense ODN in the understanding of renal and vascular pathophysiology, as well as in experimental therapeutic protocols. Recent improvements in the design of RNA molecules with modified properties will be addressed first. Second, the specificity of how antisense ODN can be used to dissect molecular mechanisms of disease and how specificity offers new possibilities of drug treatment is discussed. Third, we will discuss the endothelium as a potential target tissue for antisense therapy. Finally, new, non-viral, gene transfer techniques that enhance ODN uptake under experimental conditions and may be useful in future therapeutic trials are presented.

Key words: gene transfer, antisense, oligodesoxynucleotides, protein kinase C, adhesion molecules, acute renal failure, endothelium.

CHEMICAL MODIFICATION OF OLIGODESOXYNUCLEOTIDE

The naturally occurring phosphodiester-linked ODN that were used initially were degraded rapidly by cellular nucleases and therefore could not be used as *in vivo* therapeutic agents. Thus, chemically modified antisense ODN were developed that are more resistant to endogenous degradation. An example of such stable analogs are oligonucleotides with a phosphorothioate modified backbone. These compounds are relatively stable and are the first generation of antisense compounds used in clinical trials [2]. However, phosphorothioates are not ideal and possess several properties that make it unsuitable for therapeutic purposes. The main disadvantages of phosphorothioates are low binding affinities for stranded RNA and double-stranded DNA targets compared to natural phosphodiesters [2], and nucleotide independent binding to a variety of cellular proteins [3]. Subsequently, DNA analogs with nonphosphodiester backbones have been developed. A larger number of derivatives are now available in which the phosphodiester linkage has been replaced but the deoxyribose structure retained. These derivatives include compounds ranging from phosphate backbone (phosphodithioates, chimeric methylphosphonate-phosphodiesters, peptide nucleic acids) and 5-propynyl-pyrimidine containing oligomers to sugar modifications (2′-substituted ribonucleosides, a-configuration) [4]. However, only a few of these structures, such as those having a thioformacetal or a carboxamide linkage, appear to be good structural DNA mimics.

Recently, a new type of a deoxyoligonucleotide analog was synthesized with a modification of the phosphate backbone, where the O3′-P bonds are replaced by N3′-P linkages [5, 6]. These so-called phosphoramidate analogs show several promising features. They have an achiral phosphorus-containing, negatively charged backbone and therefore exhibit good water solubility. In

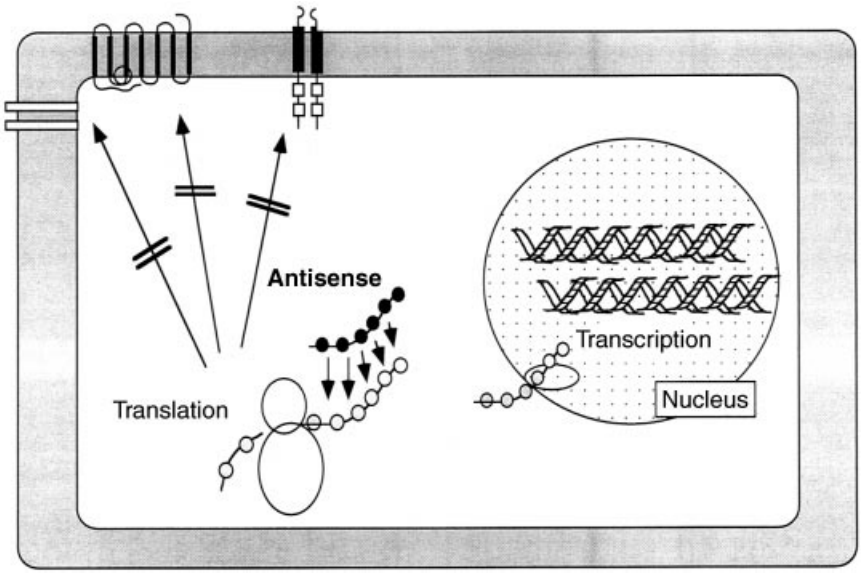

Fig. 1. Schematic diagram of antisense oligodesoxynucleotide (ODN)-mediated effects on protein expression.

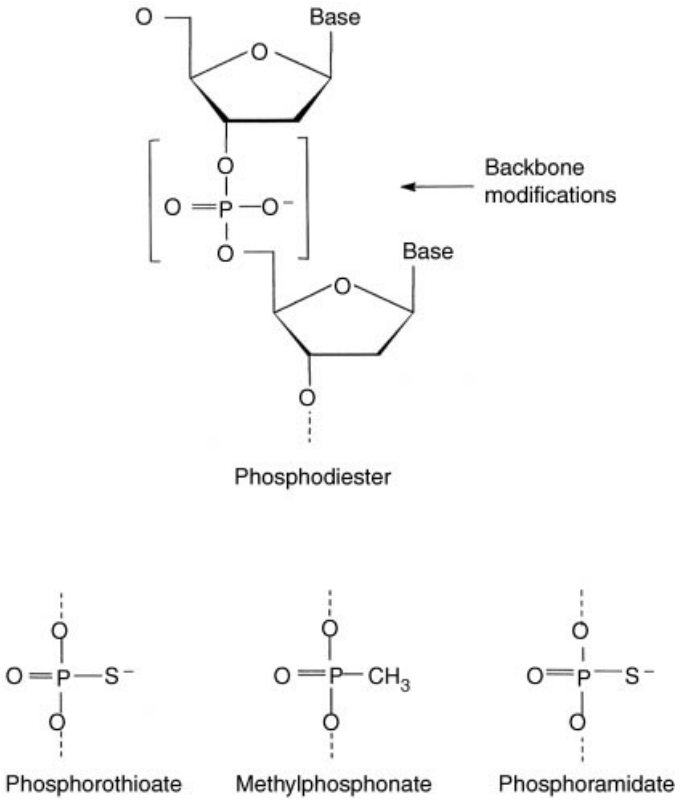

Fig. 2. Backbone modifications of oligodeoxynucleotide (ODN; see text).

addition, they feature improved resistance to nuclease degradation. Thus, they form very stable sequence-specific duplexes with single-stranded DNA, RNA, and with themselves. The phosphoramidate analogs are also able to form stable triplexes with double-stranded DNA and RNA under nearly physiological conditions [7]. Phosphoramidates are also more digestion resistant and display less protein binding than phosphorothioates [8, 9]. Figure 2 shows a schematic of ODN backbone modifications. A major problem is the large scale synthesis of these compounds. We have focused our efforts on the synthesis of phosphoramidate ODN with a method that employs a phosphoramidite amine-exchange reaction [10]. This method utilizes the corresponding monomethoxytrityl-protected 3′-amino-2′,3′-dideoxynucleoside-5′-phosphoramidites as building blocks.

SPECIFICITY

Antisense ODN are directed against distinct molecular entities. Thus, the effective therapeutic use of antisense ODN offers the possibility to directly interfere with the molecular mechanisms of the pathophysiological process [11]. Such an approach allows very specific hypothesis testing. The specificity of the approach enabled us to directly target molecules with similar properties and structure such as kinase isoforms from the same kinase family, small GTP-binding proteins, or subtypes of receptors. We have used the unique property of antisense treatment to dissect the function of the various protein kinase C (PKC) isoforms in endothelial cells. PKC is a group of calcium and phospholipid-dependent protein kinases (isoforms) with a broad substrate specificity. PKC isoforms are involved in signal transduction responses. The enzyme family was first described by Nishizuka and colleagues [12]. PKC is ubiquitously distributed and plays an important role in the control or regulation of many different biological processes [12]. In endothelial cells, PKC has been implicated in the expression and regulation of adhesion molecules [13], in the expression of endothelin-1 [14], and in the proliferative response to hormones and growth factors [15]. Furthermore, endothelial PKC appears to mediate the intracellular effects of shear stress [8] and may also be important to angiogenesis [16]. Investigating PKC is difficult because PKC is not a single entity, but consists of several distinct isoforms with different regulatory and biochemical properties [13]. The PKC isoforms are expressed on separate genes and may play different roles in cell signaling and cell function [12].

Presently, the mammalian PKC family consists of 12 different polypeptides: α, βI, βII, γ, δ, ϵ, ζ, η, θ, τ, λ and μ. An analysis of isoform expression and distribution is necessary to investigate

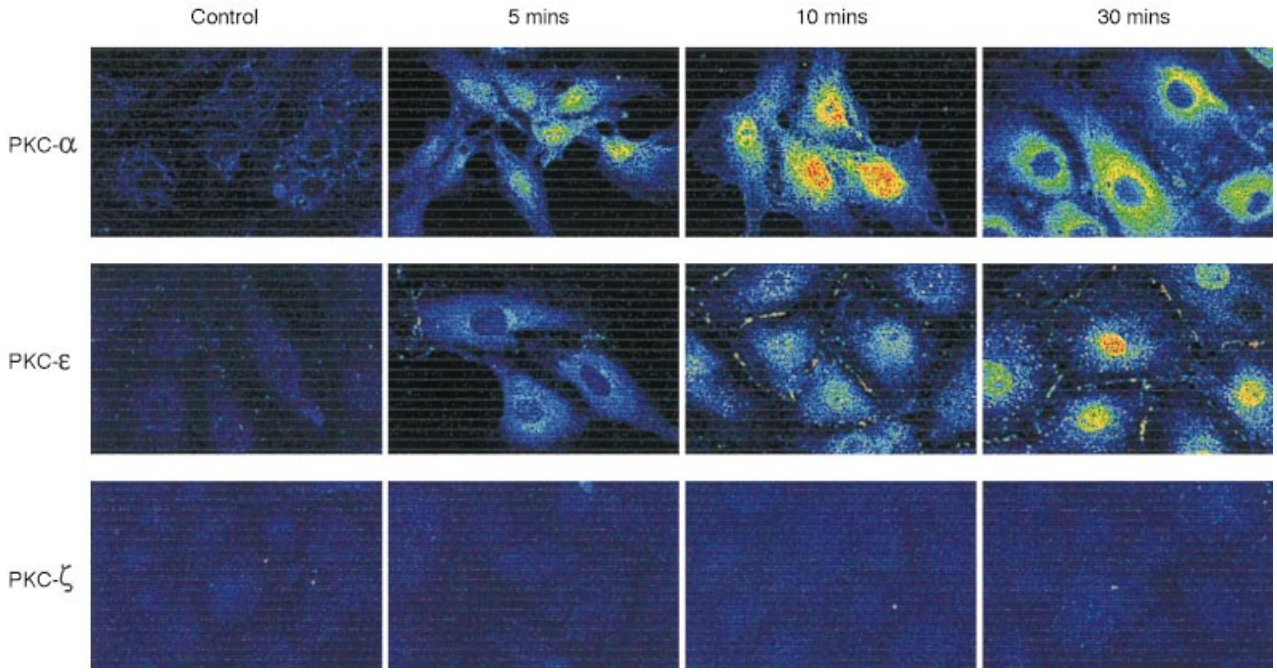

Fig. 3. Effect of high glucose (20 mM) on the intracellular distribution of protein kinase C (PKC) isoform α, ε, and ζ under control conditions (left panel, control), and after 5, 10 and 30 minutes. High glucose induced changes in intracellular distribution of PKC isoform α and ε. In contrast, PKC ζ was not influenced by high glucose. The graded color bar indicates different PKC concentrations whereby blue, green, yellow and red represent increasing PKC concentrations, respectively. The publication of this figure in color was made possible by a grant from Perkin Elmer, Applied Biosystems Division, Foster City, California, USA.

PKC's role in signaling. Since PKC plays an important role in the intracellular signal transduction pathways of the endothelium and is involved in various functions of the endothelium such as expression of adhesion molecules and regulation of the permeability barrier, defining which PKC isoform mediates a specific signal is of considerable interest. However, thus far no specific inhibitors for the different PKC isoforms are available.

This problem would appear ideal for an antisense ODN-based approach. We observed that hyperglycemia increased endothelial cell-layer permeability via a PKC-mediated mechanism. To determine which PKC isoform was responsible, we relied on an antisense strategy. We concentrated on the PKC isoforms α and ε, because our confocal immunofluorescent data implicated those isoforms. These data are reviewed in Figure 3. We included antisense to PKC ζ as an additional control. An antisense ODN (ISIS 3521) was selected against the human 3′-untranslated region derived from the human PKC α sequence (European Molecular Biology Laboratories data base, Heidelberg, Germany). The antisense sequence used for PKC α was (5′ GTT.CTC.GCT.GGT.GAG.TTT CA 3′). The sense ODN sequence (5′ TG.AAA.CTC.ACC.AGC.GAG.AAC 3′), a reverse ODN sequence (5′ AC.TTT.GAG.TGG.TCG.CTC.TTG 3′) and a scrambled version (5′ GAG.TTG.CTT.GCT.TAT.CGG.TC 3′) were used as controls. The antisense sequence used for PKC ε against the human AUG start codon was (5′ GCC.ATT.GAA.CAC.TACCAT 3′).

Figure 4 shows a Western blot analysis of PKC α, PKC ε, and PKC ζ after transfection with antisense ODN. Antisense ODN led to a down-regulation of the respective PKC isoforms to 40 to 30% as compared to control. In contrast, protein levels of PKC ε were not affected by exposure of the endothelial cells to antisense ODN against PKC α.

These antisense ODN were used to influence the glucose-induced increase in endothelial cell permeability, as shown in Figure 5. Antisense ODN for PKC α almost completely inhibited the increase in glucose-induced endothelial cell permeability. Sense and scrambled ODN for PKC α had no effect on the glucose-induced permeability. In contrast to the effects of antisense against PKC α, the antisense ODN against PKC ε did not reduce the glucose-induced permeability significantly. This experience demonstrates that an antisense ODN approach can be used for delineation of the specific PKC effects in signal transduction and cell physiology. Early experiments have shown that this approach can also be used *in vivo*. Intraperitoneal injection of ODN in mice caused a dose-dependent, ODN sequence-dependent reduction in PKC α mRNA [17]. Thus, this approach may possibly be applied to prevent glucose-induced vascular changes *in vivo.* We are presently using this approach for the treatment of diabetes-induced changes in the rat.

The utility of antisense to block intracellular signaling processes is of course not limited to PKC. Other examples include antisense directed at inhibiting the production of GTP-binding proteins, other kinases and transcription factors. Several groups have shown that antisense against cdc2 kinases exert specific effects in vascular tissue [18, 19]. Nikajima et al have used antisense ODN for the inhibition of NFκB [20]. In addition, antisense can also be used to investigate subtypes of membrane-bound receptors, such as the FGF receptor family [21, 22] or the rapidly expanding family of VEGF receptors [23]. Still other examples include targeting of proteases or components of the cell cycle [24–26].

Fig. 4. Western blot analysis of antisense oligodesoxynucleotide (ODN) against protein kinase C (PKC) α, ε and ζ in endothelial cells. Western blots were stained with PKC specific antibodies as indicated. Antisense ODN against PKC α led to a significant downregulation of PKC α, while PKC ε and ζ protein levels were not influenced. Antisense ODN against PKC ε and PKC ζ, respectively, also led to a specific inhibition of PKC isoform expression without influencing the protein levels of the other PKC isoforms.

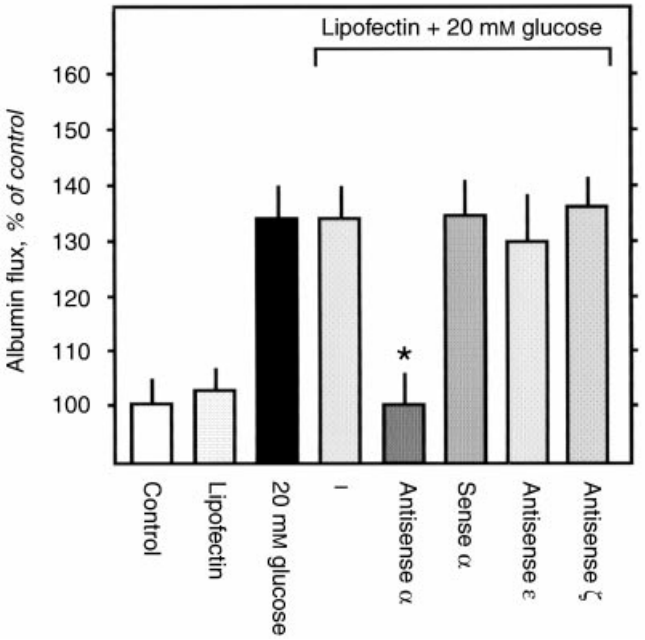

Fig. 5. Effect of antisense oligodesoxynucleotide (ODN) against protein kinase C (PKC) α, sense, scrambled ODN, or antisense against PKC ε and ζ on glucose (20 mM)-induced endothelial cell permeability. Endothelial cells were exposed to ODN with lipofectin (10 mg/ml) 24 hours before exposure to 20 mM glucose. Antisense against PKC α significantly reduced the glucose-induced permeability ($P < 0.01$), while the control sense and scrambled ODN, the ODN against PKC ε and PKC ζ, or lipofectin alone, had no significant effect.

TARGETING

Identifying potential targets for antisense strategies is easy; however, getting the antisense to the targets can be insurmountably difficult. Fortunately, since antisense ODN are small molecules they cross the cell membrane more easily than other genetic material. When injected into the venous circulation, antisense ODN demonstrate a rather high first pass effect and are rapidly taken up by the liver. However, about 1 to 3% of the antisense ODN reaches the coronary circulation, and between 0.5 and 1% arrives in the kidney after intravenous injection. Our own experiments show that the addition of cationic lipids considerably increase the percentage of renal uptake. The uptake into the renal vasculature takes place within minutes. Most of the circulating ODN are taken up by the endothelium. In our experience, even the intimal layers of vascular cells are not reached by circulating antisense ODN. However, other groups have observed an antisense effect in media vascular smooth muscle cells [27]. Conceivably, injured vascular tissue or damaged enothelial cells may exhibit altered uptake characteristics. In addition, cells adjacent to the endothelium and not shielded by the basal lamina, such as mesangial cells in the kidney, may also take up circulating ODN [28]. The rapid uptake by the endothelium makes this tissue a suitable target for antisense. The endothelium may be an interesting target for gene therapy, because endothelial cells play a major role in the development of all chronic vascular and renal diseases.

Adhesion of leukocytes to the endothelium plays an important role in such diverse processes as inflammation, transplantation, and atherosclerosis. Reperfusion injury involves activated leukocytes with enhanced adhesiveness to endothelium [29]. Adhesion

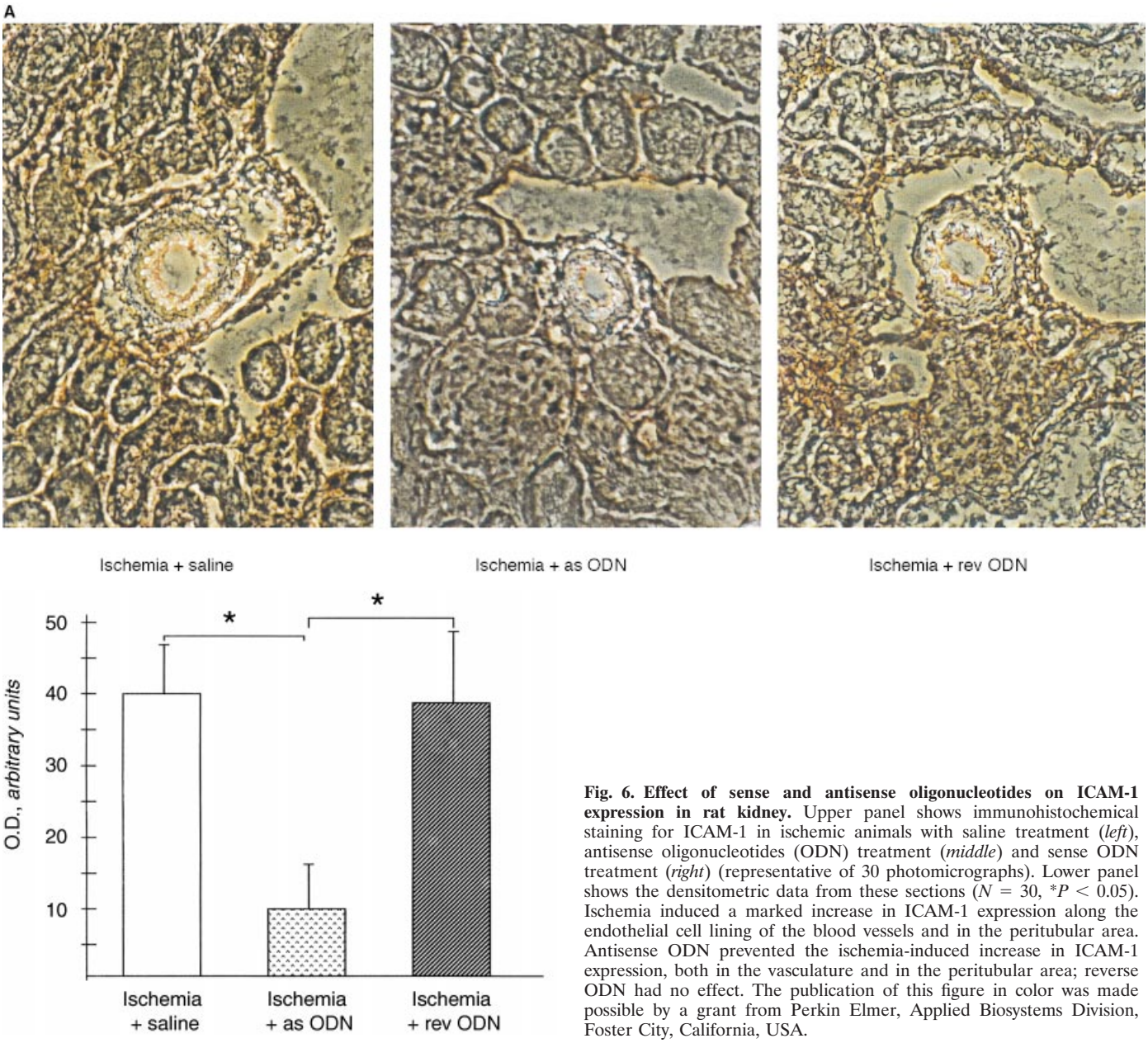

Fig. 6. Effect of sense and antisense oligonucleotides on ICAM-1 expression in rat kidney. Upper panel shows immunohistochemical staining for ICAM-1 in ischemic animals with saline treatment (*left*), antisense oligonucleotides (ODN) treatment (*middle*) and sense ODN treatment (*right*) (representative of 30 photomicrographs). Lower panel shows the densitometric data from these sections ($N = 30$, $^*P < 0.05$). Ischemia induced a marked increase in ICAM-1 expression along the endothelial cell lining of the blood vessels and in the peritubular area. Antisense ODN prevented the ischemia-induced increase in ICAM-1 expression, both in the vasculature and in the peritubular area; reverse ODN had no effect. The publication of this figure in color was made possible by a grant from Perkin Elmer, Applied Biosystems Division, Foster City, California, USA.

molecule mediated, neutrophil endothelial binding is inherent to this process [30]. The leukocyte $\beta 2$ integrin complex (CD11/CD18) interacts with the endothelial ligand intercellular adhesion molecule-1 (ICAM-1). The initial rolling of neutrophils is mediated by the selectins, while CD11/CD18-ICAM-1 interactions are responsible for leukocyte adhesion and diapedesis [31]. Studies in liver, brain, and myocardium showed that ICAM-1 is up-regulated during ischemia-reperfusion [30]. Antibodies against either CD11/CD18 or ICAM-1 prevented tissue damage and protect organ function in other studies [32–34]. We used antisense ODN for ICAM-1 to influence the expression of adhesion molecules and to prevent reperfusion injury in the ischemic kidney of the rat. Phosphorothioate oligodeoxyribonucleotides (ODN) were used and an antisense ODN (ISIS 1939) against the human 3′ untranslated region derived from the rat ICAM sequence RSICAM and the human ICAM-1 sequence HSICAM01 (European Molecular Biology Laboratories data base) selected [35, 36]. For the rat experiments, we compared rat and human sequence data and used the rat homologue to ISIS 1939 (5′ ACC GGA TAT CAC ACC TTC CT 3′). The reverse ODN sequence was used as control. As in the previous experiments, a cationic lipid was used solution to enhance ODN uptake.

From preliminary *in vitro* experiments, a lipofectin concentration of 0.8 mg/mg DNA and a ODN concentration of 2 mg/kg body wt was chosen for the *in vitro* studies. Figure 6 shows the effect of reverse and antisense oligonucleotides on expression of ICAM-1 in renal cortical vessels 24 hours after 30 minutes of ischemia. The saline injected control showed ICAM-1 staining along the vascular intima. This staining was decreased in antisense ODN treated animals. The reverse ODN treated animals, on the

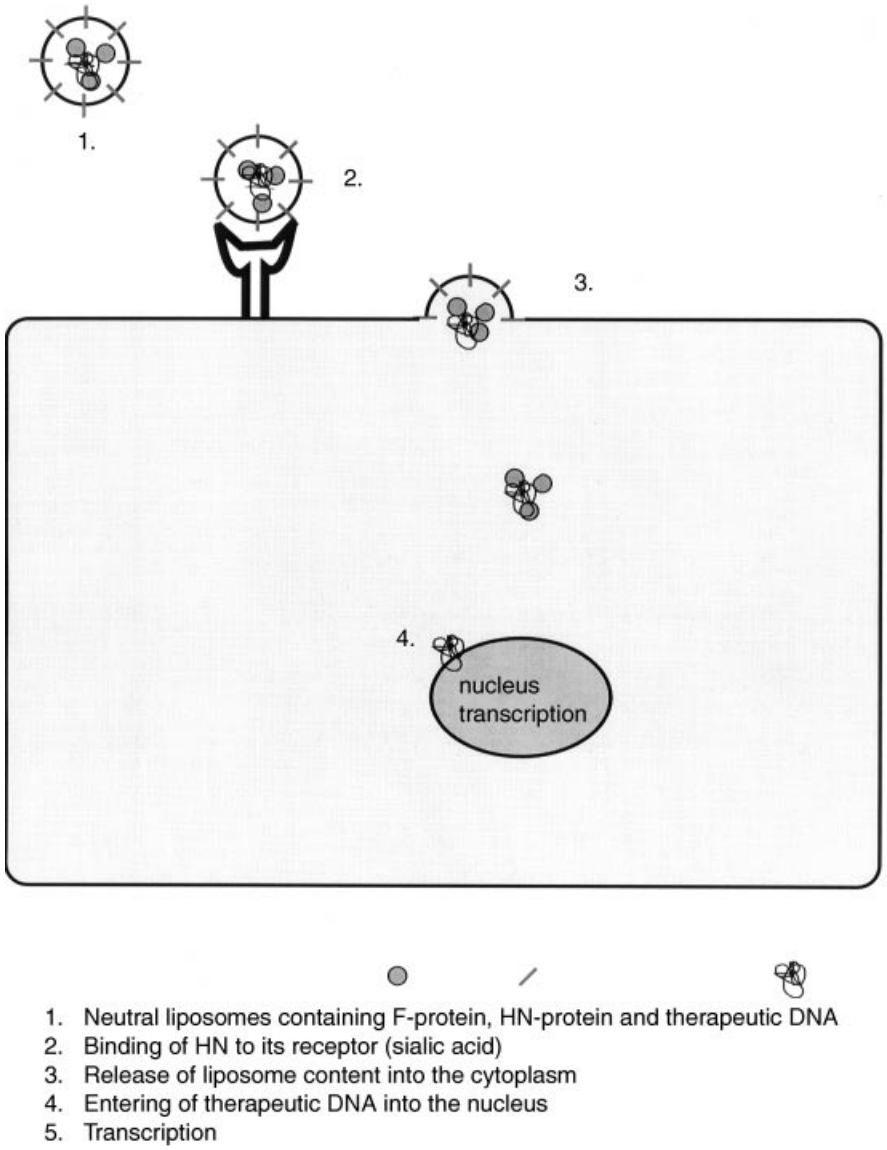

Fig. 7. Schematic diagram of transfection using liposomes containing F-protein, HN-protein and DNA. (*1*) Neutral liposomes containing F-protein and HN-protein from Sendai virus as well as therapeutical DNA are loaded for gene therapy approaches. (*2*) The HN binds to its receptor sialic acid. (*3*) The liposome content is released into the nucleus. (*4*) The therapeutic DNA enters into the nucleus and transcription begins.

other hand, showed prominent ICAM-1 staining. In antisense treated animals, renal function was preserved and perivascular leukocyte infiltration was inhibited.

We believe that the antisense approach to acute renal failure and reperfusion injury could have great clinical utility. We do not envision prophylactic preoperative antisense ODN treatment in patients. Instead, we believe such an approach would be both more valuable and practical in transplantation medicine. For instance, cadaveric donor kidney are routinely stored in Collins or similar solutions for 12 to 72 hours before transplantation. Delayed graft function from ischemia and reperfusion injury is a major post-transplant problem and has a direct negative impact on long-term graft survival [36]. Transplanted hearts and livers also are subject to reperfusion injury and ICAM-1 seems to play a role in acute and chronic rejection [37–40]. The antisense ODN treatment is not subject to the same immunological problems that accompany the use of antibodies directed against adhesion molecules. We envision a multiple antisense ODN treatment of transplant grafts directed against a variety of adhesion molecules associated with reperfusion injury.

TRANSFER

Our experiments demonstrate that antisense ODN can be successfully used for the treatment of endothelial cells disorders. However, a limiting step in these investigations is still the low uptake of ODN in the endothelium. Relatively high concentrations of ODN have to be injected in order to achieve a significant down-regulation of the targeted protein. More effective gene transfer techniques may reduce the costs of antisense ODN therapeutic approaches, and may help to target cells within the vascular wall or other organs. For gene transfer two main approaches, viral gene transfer and non-viral techniques, have been used. Viral gene transfer techniques show high efficiency, but potentially cause viral infection, activation of oncogenes and autoimmune response. Newman et al showed that adenovirus-mediated gene transfer into rabbit arteries results in prolonged

vascular cell activation, inflammation and neointimal hyperplasia [41]. Liposome vectors contain no viral sequences and possess the desired safety profile [42, 43]. However, the liposomal approach routinely results in efficiencies below 1% [43, 44]. Several techniques have been put forward to enhance gene uptake by liposomes. Improvement of the liposomal method was demonstrated by Kaneda et al [45], who prepared DNA-loaded liposomes together with gangliosides and inactivated Sendai virus particles.

Several reports have recently demonstrated that Sendai virus-coated liposomes can mediate transfer of DNA, antisense ODN, and double stranded DNA as a decoy. An HVJ-liposome can encapsulate DNA up to 100 kbp [46]. Using HVJ-liposomes associated with gangliosides and the nuclear protein HMG-1 (non-histone chromosomal protein, high mobility group 1), Kaneda et al successfully introduced the entire human insulin gene into adult rat liver. The transcript amount of the insulin gene co-introduced with HMG-1 was more than 10 times greater that of the gene co-introduced with bovine serum albumin alone. *In vivo* insulin gene expression was also possible [46]. Tomita et al used the same approach in introducing a reporter gene into rat kidney. In 1993, Isaka et al demonstrated that transforming growth factor-beta (TGF-B) or platelet-derived growth factor-beta (PDGFB) cDNA can be transported into rat kidney with HVJ-liposomes to induce glomerulosclerosis.

The HVJ-liposome method of gene transfer has also been used successfully in the cardiovascular system. For instance, the cDNAs of the angiotensin converting enzyme (ACE) and renin genes were transfected into cultured vascular smooth muscle cells *in vitro* as well as into rat carotid artery in organ culture [47]. Morishita et al measured increased ACE activity after transfection of ACE into intact rat carotic arteries. They also demonstrated that high levels of atrial natriuretic peptide (ANP) was secreted by cultured endothelial cells [47]. HVJ-liposomes loaded with eNOScDNA restored eNOS expression in the vessel wall and inhibited neointimal vascular lesion after balloon injury [48].

The HVJ-liposome mediated transfer is also applicable for antisense ODN delivery. For instance, this delivery system was also used for antisense ODN directed at cdk 2 kinase oligonucleotides. The cyclin-dependent kinase is activated in the rat carotid artery after balloon angioplasty injury and is probably responsible for smooth musle proliferation. Morishita et al showed that intimal hyperplasia after vascular injury is inhibited by specific antisense oligonucleotides [49]. Furthermore, a gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo* [50].

A major disadvantage of this promising gene transfer approach is the tedious and time consuming preparation of the viral particles. Thus far, no preparation that would pass GMP criteria for human use has been demonstrated. We have therefore started to develop a gene delivery system containing neutral liposomes and *recombinant* viral surface fusion proteins. Yeagle reported that a fusion peptide, isolated from the remainder of F1, destabilizes membranes [51]. Therefore, he hypothesized that contact between the a hydrophobic sequence of the fusion peptide of F and the target membrane is capable of substituting for bilayer-bilayer contact. The fusion process differs from the receptor-mediated endocytosis with fusion of endosomes and lysosomes at acidic pH used by influenza virus [52]. A schematic diagram of transfection with liposomes outfitted with viral fusion proteins is shown in Figure 7.

Expression of recombinant viral fusion proteins is not an easy task. Pomaskin, Veit and Schmidt used a baculovirus system and found incomplete processing and membrane transport of F-protein, due to the different glycosylation and protein transport pathways in invertebrate cells [53]. Construction of recombinant vaccinia virus expressing Sendai-F-protein resulted in a biologically inactive protein [53], probably because the severe cytopathic effects of vaccinia virus disturbs the expression of the activity. Using PCR-mutagenesis we added a factor Xa cleavage site into the F-cDNA to obtain the correctly processed and active F-protein. For future purification of the protein we fused a histidine sequence coding at the end of the F- and HN-cDNA. The vector pcDNA3 carries the correspondent cDNAs under the control of the cytomegalovirus promoter. Furthermore, we cloned our constructs into the vector pzeoSV2. Genes cloned into pZeoSV2 are expressed from the Simian virus 40 early promoter for high level transient and stable expression in mammalian cells. Then, as shown in Figure 7, it should be possible to use the purified proteins to generate fusion protein coupled liposomes. Our method differs from commonly used HVJ-liposomes because we do not use the whole inactivated Sendai virus. Instead, we integrate the two recombinant proteins from Sendai virus into the liposomes. *In vivo* experiments will show whether these constructs will diminish cell toxicity, immune response and inflammation.

PERSPECTIVES

Antisense strategies have been termed the "poor man's road to gene therapy." The ODN have been made more robust by means of a phosphoramidate backbone. The tremendous specificity of antisense ODN was demonstrated in the PKC experiments conducted by our group, as well as in a host of studies by other investigators. Since the inhibition of mRNA transcription by antisense ODN is transient, the therapeutic potential may be limited. However, our approach to use antisense to inhibit the development of reperfusion injury and thereby delayed graft function in transplanted kidneys may be an ideal clinical setting to use antisense ODN. As in all "gene therapy" strategies, delivery is a serious problem. We believe that eventually, liposomes coated with viral fusion proteins will offer an acceptable, highly efficient delivery system. In that way, the poor may indeed inherit at least a part of the earth.

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