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Gene therapy via inducible nitric oxide synthase: a tool for the treatment of a diverse range of pathological conditions

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Abstract

Nitric oxide ($NO^{\cdot}$) is a reactive nitrogen radical produced by the NO synthase (NOS) enzymes; it affects a plethora of downstream physiological and pathological processes. The past two decades have seen an explosion in the understanding of the role of $NO^{\cdot}$ biology, highlighting various protective and damaging modes of action. Much of the controversy surrounding the role of $NO^{\cdot}$ relates to the differing concentrations generated by the three isoforms of NOS. Both calcium-dependent isoforms of the enzyme (endothelial and neuronal NOS) generate low-nanomolar/picomolar concentrations of $NO^{\cdot}$. By contrast, the calcium-independent isoform (inducible NOS (iNOS)) generates high concentrations of $NO^{\cdot}$, 2–3 orders of magnitude greater. This review summarizes the current literature in relation to iNOS gene therapy for the therapeutic benefit of various pathological conditions, including various states of vascular disease, wound healing, erectile dysfunction, renal dysfunction and oncology. The available data provide convincing evidence that manipulation of endogenous $NO^{\cdot}$ using iNOS gene therapy can provide the basis for future clinical trials.

Introduction

Since it was elucidated that the role of nitric oxide ($NO^{\cdot}$) was identical to that of endothelial-derived relaxing factor (Furchgott & Zawadzki 1980; Ignarro et al 1987), much research has focused on the physiological and pathological actions of $NO^{\cdot}$. Broadly speaking, $NO^{\cdot}$ exerts its effects in one of four ways: as an intracellular signalling molecule, as an extracellular messenger molecule, as a neurotransmitter or as a cytotoxic species. The generation of $NO^{\cdot}$ within endothelial cells of the vasculature has long been recognized to control vascular smooth muscle tone (Lincoln et al 1996) through the activation of soluble guanylate cyclase, and subsequent downstream effects on calcium regulation (Lee et al 1997; Sauzeau et al 2000). Within the central nervous system, motor neurones of the parasympathetic branch of the autonomic nervous system use $NO^{\cdot}$ as a neurotransmitter, controlling peristalsis and erectile function (Basu & Ryder 2004; Rosen & Kostis 2003). Within the immune system, output of large quantities of NO is normally attributable to the expression of inducible nitric oxide synthase (iNOS) within macrophages. As high levels of macrophage infiltration are commonly observed at transplant sites, it is accepted that $NO^{\cdot}$ produced from macrophage invasion during an immune response acts as the main cytotoxic effector molecule involved in graft rejection (Holan et al 2002).

However, this is by no means an exhaustive overview of the role of $NO^{\cdot}$ under normal or disease conditions. A brief review of the current literature may prove more confusing than beneficial, as many authors now recognize that the effects of $NO^{\cdot}$ not only depend on cell type, but are, perhaps more importantly, dose dependent. This review summarizes the most commonly used vector technologies available for efficient transgene expression, with a specific focus on iNOS gene therapy in relation to its clinical application for the treatment of a range of pathological conditions.

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Gene delivery technologies

The Achilles heel of gene therapy is efficient delivery of the gene to the site of interest. Sustained high-level transgene expression specifically at the point of interest is the ultimate goal for groups developing new means of transgene delivery. The first successful transfer of mRNA and DNA to form the first transgenic mouse model was developed by Gordon et al in 1980, and within a year they had reported the stable integration of transgenes injected into mouse pronuclei (Gordon & Ruddle 1981; Gordon et al 1980). The vectors most commonly used for gene transfer currently are classed as being of either viral or non-viral origin. Early clinical trials of both viral and non-viral vectors have produced some success, particularly in gene therapy for cancer (Caplen et al 1995; Roth et al 1996). Transfection efficiencies are unquestionably superior with viral vectors than with their non-viral counterparts. However, serious immunogenic concerns associated with the use of viral vectors still exist.

Viral vectors

A number of viruses have been developed for gene transfer, but the main interest has focused on retroviruses, adenoviruses (AdV), adeno-associated viruses (AAV) and lentiviruses.

Retroviral vectors Retroviruses are a group of viruses whose RNA genome is reverse transcribed into double-stranded DNA within infected cells. Transfected DNA then becomes incorporated into the host-cell genome. Retroviruses contain specific enhancer/promoter regions located within viral long terminal repeats. Recombinant retroviruses are developed by replacing viral genes vital for replication (gag, pol and env) with therapeutic genes (Danos & Mulligan 1988; Miller 1990). In a recent study, a retrovirus was encapsulated with a genetic segment containing the genes for both interleukin 2 and herpes simplex virus-thymidine kinase (HSV-*tk*) (Barzon et al 2003). Whilst the former gene has been well proven to evoke an anti-tumour immune response (Caruso et al 1996; Saudemont et al 2002), the latter gene is a known suicide gene, which is used to activate the pro-drug ganciclovir (Fillat et al 2003). This method of combination therapies is becoming increasingly popular, and in this case a 3–4-fold reduction in tumour size was reported (Barzon et al 2003). Further enhancement of this approach has involved co-expression of two suicide genes in a retrovirus, in this case the HSV-*tk* gene and the gene for the cytokine tumour necrosis factor-α (TNF-α). The combination of suicide transgenes significantly increased anti-tumour and pro-immune effects over HSV-*tk* or TNF-α retroviruses alone (Zhang et al 2006). Retroviruses also display important tumour-targeting properties, considered to be one of the most important issues in gene therapy. Retroviruses will only infect actively dividing cells, during mitosis, which acts as a natural selection mechanism for tumour cells. However, most tumours contain central hypoxic regions where non-dividing cells, which are locked in G_0, can escape therapy.

In order to address these problems, new retroviral vectors – replication-competent retroviruses – were developed and engineered to replicate specifically in targeted neoplastic tissues, thereby increasing the non-toxic transduction ability (Solly et al 2003).

Adenoviruses In 2004, 40% of cancer gene therapy trials involved the use of AdV vectors, making them second only to retroviruses for transgene expression models. Recombinant AdV have the advantage of providing an extremely high capacity for transgene insertion in a transient manner suitable for cancer gene therapy, as well as the ability to infect actively dividing cells and quiescent cells more commonly found within hypoxic regions of a tumour cell mass (Relph et al 2005). AdVs are double-stranded linear DNA viruses, which are normally 30–35 kb in length, and their genome is organized into five early, four intermediate and one late transcriptional units. Foreign transgenes can be incorporated into the AdV genome in at least three locations within the early transcriptional units, namely E1, E3 and E4 (Majhen & Ambriovic-Ristov 2006). The first generation of AdVs were still able to replicate at very low levels, thereby inducing a cellular immune response. This is the main problem associated with use of AdVs. Second- and third-generation AdV vectors have the transcription units E1, E2 and E4 removed, reducing their potential for an immunogenic response (Amalfitano et al 1998; Lusky et al 1998; Moorhead et al 1999). Fourth-generation AdVs are not solely AdVs, but rather hybrid combinations of AdV vectors and AAVs. These are completely devoid of any viral genes except for inverted terminal repeats isolated from the AAV (Lieber et al 1999, Lundstrom 2003). Even though fourth-generation AdVs pose no immunogenic threat, their use as a means of delivering therapeutic anti-cancer genes is limited, as the transgene is integrated into the genome of the host cells, producing prolonged high levels of transgene expression.

Adeno-associated viruses (AAVs) AAVs are members of the parvovirus family; these contain single-stranded DNA that requires a helper virus such as an adenovirus for replication. Wild-type AAV is able to infect non-dividing human cells and incorporate stably into the genome at a specific locus on chromosome 19 (Samulski 1993). Perhaps one of the most significant aspects of AAV transfection is that no pathology has been shown to occur as a result of the virus, and incorporation into the aforementioned site does not encode for any important genes.

As with the other viral vectors, recombinant AAVs (rAAVs) are formed by replacing wild-type protein-coding regions (namely rep and cap) with therapeutic genes, along with the virus's terminal repeats, which are necessary for viral integration and replication (Rolling & Samulski 1995). Unfortunately, as with all of the other potential vectors, rAAVs have their limitations. First, in contrast to the wild-type, the recombinant version of the virus loses its ability to integrate specifically (Ferrari et al 1996). Second, rAAVs can only accommodate a therapeutic DNA gene insert of up to 5.2 kb. Tu et al (2005) demonstrated that an rAAV carrying a mutant survivin gene induced sensitivity to the chemotherapeutic agent 5-fluorouracil, inhibited angiogenesis and induced apoptosis in a range of human colon cancer cell lines.

Phase I clinical trials using AAV vectors are just beginning, and the utility of AAV-based vectors for human applications remains to be determined.

Lentiviruses Lentiviruses include a variety of cross-species viruses, including viruses from primates (e.g. human and

simian immunodeficiency viruses), and non-primate viruses, including feline and bovine immunodeficiency viruses. These viruses also have the ability to incorporate into the host genome of non-dividing cells (Bukrinsky & Haffar 1997), making them particularly interesting in the potential gene therapy treatment of neurones, haematopoietic stem cells, myocytes and macrophages. Lentiviruses face the same safety concerns as the other viral vectors, as well as problems associated with large-scale production of the virus. However, many feel that if these obstacles can be overcome, lentiviruses have the most potential of the viral vectors for efficient and safe gene therapy treatments.

Non-viral vectors

Because of the problems associated with viral gene transfer, some groups have shifted their attention to the use of non-viral gene delivery. The main advantages of a non-viral transfection approach are: elimination of the potential for an immune response; practically unlimited target gene insert size; and the relatively cheap and easy large-scale production of plasmid DNA through well-established DNA isolation techniques.

Naked DNA Naked DNA has been injected directly into mouse muscle in-vivo (Wolff et al 1990). Another alternative is to attach the DNA to gold particles and then bombard the therapeutic gene into the tissue. Although these approaches have been shown to produce sustained levels of gene expression in-vivo, levels of protein expression tend to be low and transfection efficiencies are poor. This technique should not be totally discarded though, as it may prove useful in developing vaccines for HIV and influenza.

Liposomes Liposomes are spherical lipid bilayers which vary in size from 50 to 1000 nm in diameter and serve as convenient delivery vehicles for gene therapy constructs (Banerjee 2001). The field of liposome development has expanded rapidly over the past 20 years with the demand for a safe means of gene delivery that lacks pathogenicity and has the capability for insertional mutagenesis (insertion of a gene into a region of the genome coding for tumour suppressor genes or oncogenes). Essentially, DNA fuses with the lipid bilayer of the liposome, which in turn interacts with the cell membrane to assist in cell entry (Felgner et al 1994). A wide range of liposomes can be engineered, varying in size, phospholipid composition and surface characteristics to suit their intended purpose. The main problem facing liposomal gene transfer is the relatively low level of gene expression compared with that achieved with viral vectors. Under in-vivo conditions when the lipo–gene complex is delivered systemically, cationic liposomes can attract serum proteins and blood cells, resulting in dynamic changes in their physiochemical properties. Liposomes need to be able to pass though capillary walls, avoid recognition by phagocytes, bind to the surface of target cells, internalize, escape from endosomes, and then find a way to the nucleus, avoiding cytoplasmic degradation. The development of successful non-viral liposomal gene-transfer mechanisms requires an in-depth understanding of the barriers faced by liposomes and developing approaches to overcome these problems (Glover et al 2005). Novel non-viral vectors with increased stability in-vivo and that avoid plasma protein binding are emerging. Furthermore, the addition of ligands for receptor-mediated uptake, endosomal disruption sequences and nuclear import signals has been shown to improve the passage of non-viral vectors through the cell into the nucleus. A group in Japan has recently demonstrated selective anti-cancer gene therapy for cells that are positive for prostate-specific membrane antigen (PSMA) (Ikegami et al 2005). Cells positive for PSMA showed a transfection efficiency of 13.2% when transfected with the anti-PMSA–liposome complex, compared with 4% for the liposome-alone control construct. The specificity of this effect was confirmed when PSMA-negative cells such as DU145 and PC-3 showed no significant difference in transfection efficiency.

iNOS gene therapy

$NO^{\cdot}$ is generated by one of three NOS enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS) and iNOS. Unlike their names suggest, nNOS and eNOS are not solely restricted to neuronal and endothelial cells; rather these are the tissue types where they were first described. It is now clear that these enzymes are present in a variety of tissue and cell types (Forstermann et al 1995; Gath et al 1996). These two isoforms of the enzyme are constitutively expressed in mammalian cells and normally produce $NO^{\cdot}$ in relatively low (picomolar) quantities in the presence of increased intracellular calcium levels. The iNOS isoform is different in that it does not require an increase in intracellular calcium to generate $NO^{\cdot}$, as calmodulin remains non-covalently bound, constituting an essential component of this isoform (Cho et al 1992). This isoform of NOS produces quantities of $NO^{\cdot}$ 100–1000-fold greater than its constitutive NOS counterparts, until substrate or cofactor availability become rate limiting. Utilization of the iNOS enzyme has formed the basis of many novel gene therapy approaches (Table 1), from providing protection against myocardial infarction to promoting strong anti-tumour effects in a range of tumour models.

iNOS gene therapy for cardiovascular disease

The endothelial cell plays an important role in the control of vascular smooth muscle tone (Lincoln et al 1996). This physiological effect occurs through NO-mediated activation of soluble guanylate cyclase, which in turn catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), the second messenger responsible for the relaxation of vascular smooth muscle. The cGMP then activates cGMP-dependent protein kinase, which induces relaxation via two pathways within endothelial cells (Pfeifer et al 1998). The kinase reduces the sensitivity of the Ca^{2+}–calmodulin complex to Ca^{2+} and at the same time inhibits the influx of Ca^{2+} through voltage-gated ion channels. The major regulatory mechanisms involved in smooth muscle contraction are phosphorylation and dephosphorylation of the myosin light chain (MLC). MLC is phosphorylated by the Ca^{2+}–calmodulin-activated MLC kinase, and is dephosphorylated by Ca^{2+}-independent MLC phosphatase (Somlyo & Somlyo 1994). This is illustrated in Figure 1. The potent

Table 1 Summary of the various preclinical in-vivo models using inducible nitric oxide synthase (iNOS)-based gene therapy

Disease	Study	In-vivo model	Delivery	Effects
Atherosclerosis	Zanetti et al 2003	Rabbit	AdV–iNOS	Relaxation of endothelium vessels in cholesterol-fed animals
Restenosis	Shears et al 1998	Rats and pigs	AdV–iNOS	Local increase in NO levels and reduction of neointimal lesions
	Muhs et al 2003	Pigs	iNOS lipoplexes	Inhibition of stent-induced neointimal lesions
	Raman et al 2006	Pigs	AdV–iNOS and CO	Protection against vascular injury response and reduction in neointimal formation
	Pfeiffer et al 2006	Dogs	iNOS lipoplexes	Inhibition of intimal hyperplasia
Myocardial infarction	Li et al 2003, 2006	Mice	AdV–iNOS	Cardioprotection
CABG	Shears et al 1997	Rats	AdV–iNOS	Suppressed allograft arteriosclerosis
Pulmonary hypertension	Budts et al 2000	Rats	Aerosolized AdV–iNOS	Lowered pulmonary arterial pressure
	Jiang et al 2006	Rats	Aerosolized AdV–iNOS	
Renal dysfunction	Miyajima et al 2001	Mice	iNOS −/− knockouts	NO has a protective role against ureteral obstruction
	Ito et al 2004	Rats	Liposomal iNOS	
Erectile dysfunction	Garban et al 1997	Rats	Liposomal iNOS	Increases in intracavernous pressure
Wound healing	Thornton et al 1998	Rats	Naked iNOS DNA	Increased collagen levels in wounds
Murine melanoma	Xie et al 1995	Mice	Stable iNOS over expressing tumour cells	Reduction in metastatic potential
Ovarian carcinoma	Xu et al 2002a	Xenograft, mice	Microencapsulated iNOS expressing cells	Inhibition of tumorigenic potential
Murine fibrosarcoma	Worthington et al 2002	Mice	Liposomal iNOS + radiation	Tumour growth delay and radiosensitization
Murine fibrosarcoma	Worthington et al 2005	Mice	Liposomal CArG/iNOS	Tumour growth delay
Colon adenocarcinoma	McCarthy et al 2007a	Xenograft, mice	Liposomal WAF1/iNOS + radiation	Tumour growth delay and radiosensitization

vasodilation effects exerted by $NO^{\cdot}$ have been attributed to a decrease in cytosolic Ca^{2+} and calcium desensitization by stimulation of MLC phosphatase (Lee et al 1997; Sauzeau et al 2000).

Atherosclerosis

The level of $NO^{\cdot}$ plays a critical role in the aetiology of cardiovascular disease. Wilcox et al (1997) examined normal and atherosclerotic human vessels to determine levels of NOS isoforms, and found that, overall, the amount of NOS present was greater in the atherosclerotic wall than in normal vessels. However, if the constitutive isoforms are compared, eNOS expression was decreased in advanced atherosclerotic lesions, in contrast to iNOS and nNOS, which were both undetectable in normal vessels but increased in early and advanced lesions (Wilcox et al 1997). Thus, vascular pathologies are often characterized by a disruption in $NO^{\cdot}$ physiology.

A major difference between normal and atherosclerotic arteries is in the isoform of NOS they express. eNOS expression is constitutive in normal tissue and is regulated by intracellular Ca^{2+} fluxes (Forstermann et al 1994). iNOS expression has been detected in atherosclerotic plaques, and is indicative of tissue dysfunction (Calver et al 1993; Buttery et al 1996). In contrast to the other NOS isoforms, iNOS produces high concentrations of $NO^{\cdot}$, which evoke instant physiological responses, providing a vasoprotective role by inhibiting leucocyte adhesion, platelet adhesion and migration of vascular smooth muscle cells (Mellion et al 1983a; Radomski et al 1987; Garg & Hassid 1989; Kubes et al 1991; Dubey et al 1995). The role of iNOS expression in this setting has not been fully elucidated, unlike the other isoforms.

eNOS gene transfer has clearly been shown to reverse endothelial dysfunction in several disease states, including hypercholesterolaemia, atherosclerosis, diabetes mellitus, hypertension and subarachnoid haemorrhage (Mozes et al 1998; Onoue et al 1998; Sato et al 2000a; Zanetti et al 2000). nNOS gene transfer corrects cholesterol-induced endothelial dysfunction (Qian et al 1999). However, the therapeutic effects of iNOS are disputed. Zanetti et al (2003) examined the role of iNOS gene transfer in carotid arteries from hypercholesterolaemic rabbits using AdV-mediated gene transfer. They detected elevated iNOS protein expression 3 days after luminal transfection of the gene, resulting in increased superoxide production and reduced vascular relaxation in hypercholesterolaemic rabbits compared with controls, where iNOS expression had negligible effects. In contrast, Hayashi et al (2004) compared eNOS and iNOS AdV-mediated gene transfer, and examined the effect of combining the two isoforms in rabbits with advanced atherosclerosis (Hayashi et al 2004). Aortic injury was induced using a catheter inserted into the femoral artery followed by balloon inflation. The rabbits were then fed a high-cholesterol diet for 12 weeks before transduction. Utilizing catheter-based delivery, high levels of expression of both isoforms was transduced, showing adenoviral efficiency. However, only eNOS gene transfer reduced the development of advanced atherosclerosis and restored

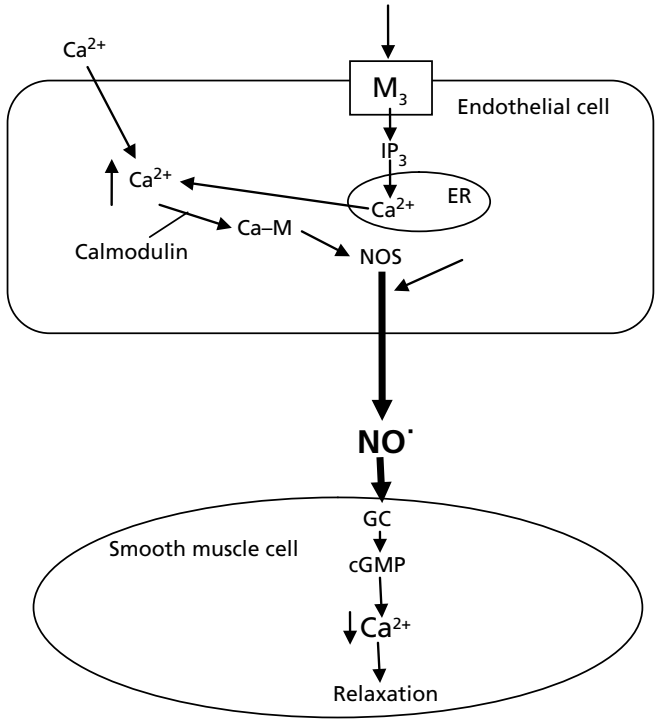

Figure 1 Mechanistic representation of nitric oxide ($NO^{\cdot}$) production leading to smooth muscle relaxation. Stimulation of the M_3 muscarinic receptor by acetylcholine causes the formation of inositol triphosphate (IP_3), resulting in the release of Ca^{2+} from the endoplasmic reticulum (ER). This, along with the influx of Ca^{2+} through calcium channels, leads to the formation Ca^{2+}–calmodulin (Ca–M) complexes. The calcium–calmodulin complex binds to the calmodulin binding site, activates the nitric oxide synthase (NOS) and produces $NO^{\cdot}$. In the smooth muscle cell the $NO^{\cdot}$ then activates the soluble guanylyl cyclase (GC), which catalyses the production of cGMP from GTP. The cGMP reduces cytosolic Ca^{2+}, leading to smooth muscle relaxation.

endothelium-dependent relaxation. Furthermore, the combination of the two isoforms had no effect, indicating that iNOS transfection actually blocked the beneficial effects of eNOS treatment on atherosclerosis. This may be due in part to rapid depletion of the substrate L-arginine and necessary co-factors such as tetrahydrobiopterin (BH_4), limiting the beneficial effects evoked by the eNOS isoform. However, under these depleted conditions, and particularly when BH_4 is limiting, the constitutive isoforms have been demonstrated to release $O_2^{\cdot-}$ (Vasquez-Vivar et al 2003; Xia & Zweier 1997).

Hayashi et al (2004) postulated that areas of deep atherosclerosis were the ideal conditions for the generation of $O_2^{\cdot-}$ from eNOS, and that any $NO^{\cdot}$ produced from the transfection of the AdV–iNOS therapy would rapidly react with the $O_2^{\cdot-}$, resulting in the production of peroxynitrite ($ONOO^-$). Analysis of atherosclerotic regions revealed elevated nitrotyrosine (a marker for $ONOO^-$), which was significantly increased in arteries pre-treated with AdV–iNOS (Hayashi et al 2004).

Restenosis

Atherosclerotic deposits are treated using percutaneous transluminal coronary angioplasty (PTCA) and, in many cases, balloon angioplasty. While this treatment reduces atherosclerotic plaques, considerable damage occurs to vascular endothelial cells. Damage to the endothelium has far-reaching consequences, which limits further treatment options such as further PTCA, bypass grafting and stenting (Ross 1990). In essence, any damage to the endothelium exposes the underlying smooth muscle cells to circulating blood (Clowes et al 1983). A cascade of events ensues, characterized by platelet aggregation, leucocyte chemotaxis, changes in the extracellular matrix, proliferation of smooth muscle cells, and migration and proliferation of endothelial cells (Fingerle et al 1989). These changes result in protrusion of the intima into the lumen, with serious vascular repercussions, such as restenosis.

$NO^{\cdot}$ has a vasoprotective role and has been shown to prevent platelet aggregation mediated by cGMP (Mellion et al 1983; Radomski et al 1987; Fingerle et al 1989). Leucocyte activation has been shown to stimulate various cytokines and growth factors that are associated with the progression of intimal hyperplasia. Furthermore, several groups have shown that the leucocyte response is abrogated in iNOS knockout mice (Kubes et al 1991; Hickey et al 1997; Muhs et al 2003). $NO^{\cdot}$ inhibits the proliferation and migration of smooth muscle cells (Garg & Hassid 1989; Guo et al 1995). Evidence also suggests that the loss of $NO^{\cdot}$ following injury could be highly significant in accelerating the thickening of neointimal lesions and indeed atherogenesis (Bucala et al 1991). So clearly $NO^{\cdot}$ has a major role in preventing intimal hyperplasia.

Shears et al (1998) performed a series of Greiss test assays in-vitro to determine the extent to which various multiplicity of infection (MOI) of the AdV–iNOS gene therapy could induce $NO^{\cdot}$ expression. Supplementing the cell culture medium with BH_4 enhanced the total nitrite expression by

3 fold. An MOI of 1 increased the total nitrite concentration by 8.9 fold (from 2.2 to 19.7 nmol mg^{-1}) over non-infected control cells. Increasing the MOI to 10 resulted in a linear increase in total nitrite to 228 nmol mg^{-1}. However, higher concentrations of AdV–iNOS infection resulted in a plateau effect. In the same study, the carotid arteries of rats and the iliac arteries of pigs were clamped and injured using a balloon catheter inflated to 5 atm for 5 min. Treatment with AdV–iNOS was then administered through an angiocatheter for 60 min and 30 min, respectively. Following balloon injury, the development of thick neointimal lesions occurs within 14 days in the rat carotid arteries and 3 weeks in pig iliac arteries. Neointimal lesions were inhibited by AdV–iNOS in carotid arteries treated with 2×10^6 plaque-forming units (PFU) and in pig iliac arteries treated with 5×10^8 PFU (Shears et al 1998). Interestingly, the effects of iNOS were completely reversed by a selective iNOS inhibitor – L-N6-(1-iminoethyl)-lysine (NIL) – indicating that iNOS is directly responsible for protection against neointimal hyperplasia and, ultimately, restenosis (Shears et al 1998).

AdV–iNOS gene therapy has also been delivered to pigs in combination with carbon monoxide (CO) (Raman et al 2006). Like $NO^{\cdot}$, CO possesses vasoprotective properties. Although $NO^{\cdot}$ and CO are structurally and functionally similar, they operate through distinct signalling pathways. Production of $NO^{\cdot}$ following iNOS gene transfer imparts its vasoprotective properties via a cGMP-independent mechanism, whereas CO confers its vasoprotective properties via a cGMP-dependent mechanism (Kibbe et al 2000; Otterbein et al 2003). Individually, both CO and $NO^{\cdot}$ delivered to injured pig iliac arteries reduced intimal formation by 38% and 46%, respectively. Combined delivery of CO and $NO^{\cdot}$ reduced intimal formation by 63%, indicating an increased but sub-additive effect (Raman et al 2006). This indicates that both possess common vasoprotective actions whilst also operating through different mechanistic pathways.

There are obvious safety issues associated with viral gene delivery, and so liposomal-based transfer has been used to deliver iNOS, with the aim of inhibiting stent-induced neointimal hyperplasia (Muhs et al 2003). Indeed, local transfer of iNOS lipoplexes has been shown to reduce femoral in-stent plaques by approximately 40%, while proliferation of smooth muscle cells was reduced by nearly 77%, indicating the clinical potential of this treatment (Muhs et al 2003). Cationic lipoplexes have also been delivered following polytetrafluoroethylene grafting, where the carotid artery of dogs was clamped proximally and distally and a balloon catheter delivered to the clamped region through which the lipoplex solution was delivered to determine transfection efficiency (Pfeiffer et al 2006). In addition, a long-term experiment in this model involved a 15 cm incision in the clamped region where polytetrafluoroethylene was inserted as a bypass graft and perfused. After 6 months all prostheses were removed and analysed. Intimal thickness was significantly reduced at both proximal and distal ends by at least 40% compared with controls (Pfeiffer et al 2006).

Currently, it is generally accepted that eNOS and iNOS gene delivery inhibits proliferation in smooth muscle cells, whilst allowing regeneration of the damaged endothelium and preventing neointimal thickening. Cooney et al (2007) compared the effects of eNOS and iNOS on both endothelial regeneration and neointimal hyperplasia after vascular injury. They found that iNOS inhibited and eNOS enhanced endothelial regeneration, although both isoforms inhibited neointimal formation. The authors suggest that the difference in endothelial regeneration between the two isoforms could be because more $NO^{\cdot}$ is produced from iNOS, which is prone to 'uncoupling', subsequently leading to superoxide generation and toxicity. However, prior in-vitro work by Cooney et al (2006) indicated that both eNOS and iNOS caused reduced proliferation, reduced angiogenesis and no induction of apoptosis in endothelial or vascular smooth muscle cells in the absence of injury, highlighting the need for thorough in-vivo investigations.

Some evidence suggests that inhibition of the migration and proliferation of smooth muscle cells following gene transfer of eNOS or iNOS is due to the increased expression of cyclin-dependent kinase inhibitors p21 and p27 (Kullo et al 1997; Kibbe et al 2000; Sato et al 2000b). However, Iwashina et al (1998) delivered the iNOS gene to smooth muscle cells and found evidence for increased apoptosis (Iwashina et al 1998).

In-stent restenosis still poses a serious clinical problem, but with only a limited arterial segment affected, iNOS gene therapy could be applied locally to prevent this condition. Again, high $NO^{\cdot}$ production from iNOS gene transfer reduces neointimal hyperplasia and subsequent restenosis, but impairs endothelial regeneration. Clearly, careful consideration has to be given when selecting the most appropriate NOS gene therapy treatment for vascular damage and associated pathologies.

Myocardial infarction

The heart can adapt to stress and this is most evident during ischaemic preconditioning. This is when periods of mild ischaemia are delivered to enhance the heart's tolerance to further ischaemic stress. There are two phases: early and late preconditioning. Early preconditioning develops in minutes and can last for a few hours; the late phase appears within 24 h and can last up to 4 days (Marber et al 1993; Bolli 1996). Late preconditioning protects against myocardial infarction and myocardial stunning and is triggered by $NO^{\cdot}$ generation (Bolli et al 1997). The isoform of $NO^{\cdot}$ involved in late preconditioning was elucidated by Guo et al (1999) when they genetically engineered iNOS knockout mice in a model of late preconditioning. They provided molecular evidence which proved that late preconditioning up-regulated iNOS. Furthermore, targeted disruption of iNOS completely abrogated cardioprotection, primarily in late preconditioning (Guo et al 1999). It is now accepted that iNOS is the specific protein responsible for late preconditioning, and can therefore be exploited for therapeutic benefit (Bolli et al 1998; Guo et al 1999).

Several groups have shown that short-term overexpression of iNOS is cardioprotective. Li et al (2003) examined whether iNOS gene transfer would confer resistance following ischaemic injury in combination with cyclooxygenase-2 (COX-2) (Li et al 2003). Replication-deficient AdV–iNOS was delivered via intramyocardial injection in a murine model. This increased cardioprotection to the magnitude observed in late

preconditioning, with a significant reduction in infarct size (by 67%) compared with controls (Li et al 2003). In addition, there was a significant increase in COX-2 protein expression 3 days after iNOS gene transfer, which could be completely abrogated using specific COX-2 inhibitors, whilst reversing the cardioprotective effects of $NO^{\cdot}$ overexpression. Therefore, the formation of a tightly bound unit between iNOS and COX-2 appears necessary in affording cardioprotection (Li et al 2003).

Bolli (2000) suggested that inhibition of COX-2 has no effect on iNOS and that COX-2 must therefore lie downstream (Bolli 2000). $NO^{\cdot}$ can directly activate COX-2 and does lie downstream; however, later studies by Li et al (2003) have shown that iNOS and COX-2 activity are directly dependent on each other (Salvemini et al 1993; Li et al 2006). Li et al (2006) then went on to examine whether long-term upregulation of iNOS could be cardioprotective. Mice received intra-myocardial injections of recombinant AdV–iNOS and then myocardial infarction was induced either 1 or 2 months later by a 30-min coronary artery occlusion, followed by 4 h of reperfusion. iNOS expression was significantly increased at both 1 and 2 months (2.8- and 2.1-fold, respectively) compared with LacZ control mice (Li et al 2006). The infarct-sparing effects of iNOS gene transfer compared with LacZ control mice were 24.2% and 23.4% at 1 and 2 months, respectively. In addition, the modified AdV vector (Av3) used to deliver the iNOS gene had deletions in E1, E2a and E3 regions, conferring long expression and negligible immune response, permitting its application in preclinical testing of iNOS gene therapy for myocardial infarction (Li et al 2006).

Liu et al (2007) finally elucidated that iNOS gene therapy is mediated by COX-2 via a nuclear factor-kappaB (NF-κB)-dependent pathway. They did this by using genetically engineered mice rather than pharmacological agents, which may not perform as consistently in-vivo. Two types of mice were used: COX-2 mutants and transgenic mice with cardiac-specific abrogation of NF-κB (Li et al 2007). The Av3 vector was used to deliver the iNOS gene. Three days after treatment, the infarct size was reduced to 42% in Av3/iNOS wild-type mice versus Av3/LacZ mice (Li et al 2007). In the COX-2 −/− mice there was no reduction in infarct size compared with controls. In addition, iNOS gene transfer increased the phosphorylation of IκBα, resulting in pronounced activation of cardiac NF-κB (Li et al 2007). Mice with the cardiac-specific NF-κB abrogation had no detectable COX-2 protein expression after iNOS gene transfer and no reduction in infarction size. This demonstrated that COX-2 and NF-κB need to be present for iNOS gene therapy to offer cardioprotection, and that NF-κB activation is necessary for upregulation of COX-2 by iNOS (Li et al 2007).

There have been many studies with varying views on $NO^{\cdot}$ and cardioprotection; specifically over the past 10 years the majority support the cardioprotective role of $NO^{\cdot}$(Vanhoutte & Scott-Burden 1994; Davignon & Ganz 2004; Kibbe et al 1999a, b). The in-vivo gene therapy studies by Li et al have clearly shown that iNOS gene therapy can reduce infarction in both the short and long term, and they have provided compelling evidence that preclinical trials are necessary to take this treatment forward. Rather than being a damaging protein, iNOS is a cardioprotective enzyme with many advantageous clinical applications (Jones & Bolli 2006).

Coronary artery bypass graft (CABG)

CABG is one of the treatment options to alleviate pain and related symptoms in patients with vaso-occlusive disease. In the year ending in March 2006 over 20000 CABG procedures were performed in the UK. Although survival rates are quoted as 98.4%, this is only for 30 days post-surgery for first-time patients (heartsurgery.healthcarecommission.org.uk). The most common reason for failure of CABG is the subsequent development of intimal hyperplasia. Many attempts have been made to prevent graft failure using calcium channel blockers, $NO^{\cdot}$ precursors or using immunosuppressant agents that have prevented arteriosclerosis in transplants (Huang et al 2001; Kown et al 2001; Walpoth et al 2001). Significant limitations are associated with systemic administration of these agents, particularly in relation to unnecessary toxicity. Treatment to prevent intimal hyperplasia should be confined to the vascular graft. When the endothelium fails to synthesize $NO^{\cdot}$, the vasoprotective properties are lost and intimal hyperplasia ensues. $NO^{\cdot}$ gene therapy seems to be an ideal strategy to prevent failure of CABG. It can be delivered locally to the graft via an intraluminal catheter, or extraluminally via polymeric gels or even ex-vivo prior to grafting. Tzeng et al (1996) found that, despite a low transfection efficiency (1%), retroviral–iNOS delivery could decrease intimal hyperplasia. Following balloon-catheter injury, the observed intimal thickening of 53% was reversed by iNOS gene therapy, although the co-factor BH_4 was also required (Tzeng et al 1996). Currently it would appear that no one isoform is superior in preventing graft failure, but iNOS does generate higher levels of $NO^{\cdot}$ which can enable the use of smaller titres of virus, potentially limiting treatment-associated toxicity or immune response (Barbato & Tzeng 2004).

Intimal thickening can contribute to rejection of arterial allografts, which accelerates the development of coronary arteriosclerosis, resulting in allograft dysfunction (Ventura et al 1993; Ardehali 1995). Shears et al (1997) examined how iNOS inhibits atherogenesis in grafts. Aortic transplantations were performed on rats, and grafts were left for 28 days before being excised and examined. Ex-vivo aortas received AdV–iNOS gene therapy for 1 h before grafting (Shears et al 1997). AdV-lacZ was used as a control to determine if iNOS gene transfer was responsible for preventing atherosclerosis. In addition, 50% of the grafts also received ciclosporin, which is a known accelerator of atherosclerosis and inhibits iNOS in-vitro (Marumo et al 1995; Shears et al 1997). Grafts treated with AdV–iNOS showed near-total suppression of allograft arteriosclerosis; similar effects also occurred in the ciclosporin and AdV–iNOS-treated grafts. Given that ciclosporin almost totally inhibits endogenous iNOS expression in-vivo, iNOS gene therapy presents a clinically relevant treatment. Graft patients may require ciclosporin as an immunosuppressant; however, with the increase in atherosclerosis that occurs as a side-effect, the benefit may not justify the possible harm. iNOS gene therapy could be delivered to grafts either ex-vivo or locally in-vivo with high $NO^{\cdot}$ expression sufficient to eliminate life-threatening allograft atherosclerosis.

Pulmonary hypertension

Pulmonary hypertension is a serious condition, characterized by high blood pressure in the arteries that supply the lungs from the heart, which become hard and narrow. As a consequence, the heart is put under immense pressure and becomes enlarged and overworked. Eventually the right ventricle weakens and heart failure ensues. Although there are various treatment options, including anticoagulants, diuretics, inotropic agents, vasodilators and lung transplants, there is no cure. One possible treatment strategy is modification of vascular tone using aerosol gene transfer. Budts et al (2000) administered 300 μL aerosolized AdV–iNOS, eNOS or RR5 (no gene) at 3×10^9 PFU for 60 min, into rat lungs. Three days after treatment, exhaled NO levels were higher in AdV–iNOS- than in AdV–eNOS-treated rats (Budts et al 2000). Exhaled NO$^{\cdot}$ levels did not return to normal physiological levels until day 10, a significant increase after just one dose. Acute and chronic hypoxia was induced 24 h after aerosolized gene treatment, for 3 and 7 days, respectively, at 10% oxygen for 30 min. Pulmonary artery pressure (PAP) was measured continuously using a silastic catheter. Under acute hypoxia, both AdV–iNOS and AdV–eNOS reduced PAP from 28 mmHg in Ad-RR5 controls to 21 and 23 mmHg, respectively (Budts et al 2000). Under chronic hypoxic conditions, AdV–iNOS-treated rats had significantly lowered PAP and reduced vascular resistance compared with Ad-RR5 and AdV–eNOS-treated rats (Budts et al 2000). This study indicates that a single aerosol delivery of iNOS results in elevated NO$^{\cdot}$ levels for at least 1 week and is more effective than eNOS in the prevention of pulmonary hypertension.

Jiang et al (2006) delivered AdV–iNOS and AdV–eNOS as an aerosol to determine the modulation of hypoxic pulmonary vasoconstriction. Likewise, this study indicated considerable gene expression after one treatment, with increased pulmonary NO$^{\cdot}$ levels. There was a 2-fold increase in exhaled NO$^{\cdot}$ from rats that were given the AdV–iNOS aerosol compared with those given Adv–eNOS (Jiang et al 2006). Vasoconstrictive agents were given to ex-vivo small and large arteries, and a three-phase response was observed in the arterial rings: a rapid contraction (c), partial relaxation (r) and a slower contraction (c′). The effects of the agents were measured as maximal change in force, tension or pressure. There were significant decreases in force, tension and pressure in large arteries during c and c′ phase in AdV–eNOS- and AdV–iNOS-treated vessels (Jiang et al 2006). In particular, iNOS overexpression gave a significantly better vascular response. No such decrease was observed in small arteries, although AdV–iNOS overexpression completely inhibited the c′ phase (Jiang et al 2006). With just one administration, iNOS gene transfer by aerosol provided a promising basis for the development of a treatment for pulmonary hypertension.

In a further study by Stanley et al (2006), the effects of iNOS overexpression on urea production were tested in relation to pulmonary hypertension (Stanley et al 2006). As iNOS and arginase compete for a common intracellular pool of L-Arg, overexpression of iNOS could reduce the production of L-ornithine and subsequently proline, polyamine and urea formation. In pulmonary hypertension, prolines and polyamines are involved in the vascular remodelling processes that are commonly observed in this disease (Thet et al 1984). Using bovine pulmonary arterial endothelial cells (bPAEC) and AdV–iNOS transfection, increased expression of NO$^{\cdot}$ decreased the levels of urea, an effect that was reversed by the addition of the NOS inhibitor *N*-(G)-nitro-L-arginine methyl ester (L-NAME) (Stanley et al 2006). Furthermore, a decrease in urea production also resulted in impaired proliferation of the AdV–iNOS-transfected bPAEC cells (Stanley et al 2006). The reduction in urea by iNOS transfection most likely occurs by limiting the supply of L-Arg available to arginase. Arginase has a negative role with respect to pulmonary hypertension. Inhibition of arginase in atherosclerotic rings increased vasodilation (Ming et al 2004). When arginase was overexpressed, there was increased proliferation in smooth muscle cells (Ignarro et al 2001). Endothelial cells from humans with pulmonary hypertension have been shown to have higher levels of arginase protein and lower NO$^{\cdot}$ production (Xu et al 2004). Again, iNOS gene therapy has provided promising results in pulmonary hypertension by increasing vasodilation, inhibiting L-Orn production, whilst also producing beneficial decreases in cellular proliferation and subsequent vascular remodelling.

iNOS gene therapy for renal dysfunction

iNOS gene therapy has been used to improve renal function in ureteral obstruction (UO). UO is characterized by decreased blood flow and potential tissue loss in the obstructed kidney resulting from interstitial fibrosis and tubular apoptosis. The disease can be congenital or may arise from ureteral stones or carcinoma. NO$^{\cdot}$ has been shown to be anti-fibrotic and anti-apoptotic in UO (Morrissey et al 1996; Miyajima et al 2001). Intrarenal pressure changes in UO can lead to tubular cell stretch (Ricardo et al 1997) and it has been shown that NO$^{\cdot}$ is produced when renal tubular cells are exposed to mechanical stretch (Miyajima et al 2000). Miyajima et al went on to examine how NO$^{\cdot}$ contributes to renal apoptosis in UO (Miyajima et al 2001). NRK-52E kidney fibroblasts were stretched mechanically at 5 s intervals for 1 min and apoptosis was measured. Analysis indicated that stretched NRK-52E had significantly higher levels of apoptosis than unstretched cells. Furthermore, addition of L-arginine (a necessary NOS substrate) reduced apoptosis, as did the NO$^{\cdot}$ donor *S*-nitro-*N*-acetyl-penicillamine (SNAP). Levels of apoptosis were significantly increased by the NOS inhibitor L-NAME (Miyajima et al 2001). It appears that NO$^{\cdot}$ plays a protective role in stretch-induced apoptosis in renal tubular cells in UO.

iNOS knockout mice have been used to investigate UO further. A left unilateral ureteral ligation was performed to mimic UO. Results obtained indicate significantly less iNOS mRNA and protein here than in the unobstructed kidney (Miyajima et al 2001). Apoptosis levels were measured in obstructed mice and were significantly greater in iNOS –/– than in wild-type mice. The iNOS –/– mice had significantly greater loss of renal tissue and greater renal tubular proliferation than wild-type mice (Miyajima et al 2001). These findings support iNOS gene therapy as a prime candidate for the treatment of UO. In-vitro studies with human embryonic kidney 293 cells indicated a transfection efficiency of at least 50% (Ito et al 2004). The iNOS gene was introduced into the

kidneys of rats by an intra-ureteral injection using liposomal delivery. The iNOS plasmid was detected by PCR 5 weeks after the injection, and NOS protein levels were elevated for at least 32 days after just one injection (Ito et al 2004). The induction of UO in rats caused a decline in glomerular filtration rate and renal blood flow, which were significantly reversed following iNOS gene transfer compared with LACZ controls (Ito et al 2004). This study clearly highlighted the protective effect of iNOS gene transfer on renal function.

iNOS gene therapy for erectile dysfunction

$NO^{\cdot}$ mediates its effects on penile erection via a mechanism that affects smooth muscle relaxation of the corpus cavernosum and by acting as a neurotransmitter within the parasympathetic branch of the autonomic nervous system. Consequently, the erection process involves all three isoforms of the NOS enzyme (Burnett et al 1992). The final step in the pathway resulting in an erection is the release of $NO^{\cdot}$ from neurones surrounding the corpus cavernosum, activating the cGMP cell signalling pathway and resulting in smooth muscle relaxation.

Erectile dysfunction is primarily caused by the inability of the corpus cavernosum to undergo complete relaxation, due either to a decrease in penile NOS activity or to the rapid degradation of cGMP by a class of enzymes called phosphodiesterases (Basu & Ryder 2004; Rosen & Kostis 2003). Several groups have developed gene therapy protocols to deliver the NOS gene to the corpus cavernosum with the specific aim of increasing penile NOS activity. Garban et al (1997) performed a series of experiments which demonstrated that age-related erectile dysfunction in rats was ameliorated by the continuous delivery of a cocktail of iNOS-related inducers (Hung et al 1995; Garban et al 1997). Furthermore, they developed a gene therapy protocol whereby the iNOS gene was cloned under the control of the constitutive cytomegalovirus (CMV) promoter and was delivered by liposomal transfer directly to the corpus cavernosum of lightly anaesthetized rats. Five-month-old rats exhibited no change in the maximal intracavernosum pressure following gene therapy treatment compared with untreated rats. However, 20-month-old rats treated with the gene therapy exhibited significant increases in maximal intracavernosum pressure, with no significant change in the mean arterial pressure (Garban et al 1997).

This clearly demonstrates that iNOS gene therapy has the potential to correct erectile dysfunction without affecting systemic blood pressure. More current and less invasive means of treating erectile dysfunction target the $NO^{\cdot}$ signalling pathway, preventing the metabolism of cGMP by phosphodiesterases. Phosphodiesterase 5 (PD5) inhibits erection by catalyzing cGMP, thereby terminating signal transduction. Sildenafil citrate works by blocking the action of PD5, such that erection is maintained for the duration of arousal.

iNOS gene therapy for wound healing

The wound repair process is associated with increased levels of $NO^{\cdot}$ (Albina et al 1990; Carter et al 1994). Elevated $NO^{\cdot}$ levels confer desirable physiological responses that promote wound healing such as angiogenesis and the proliferation and migration of endothelial and epithelial cells (Leibovich et al 1994; Ziche et al 1994; Benrath et al 1995; Morbidelli et al 1996; Noiri et al 1996). The systemic administration of arginine has been shown to improve wound healing by providing increased substrate levels for the production of $NO^{\cdot}$ (Barbul et al 1990). Shi et al (2000) developed an in-vivo wound-healing model in which animals were given dietary supplements of arginine. Arginine supplementation increased the wound breaking strength by 55% and sponge collagen deposition by over 100%, indicating substantial improvements in wound healing. In addition, a 44% increase in wound breaking strength was observed when arginine was injected i.p. into diabetic rats (Shi et al 2003).

Further studies have shown that NOS inhibitors applied either topically or systemically can significantly retard the wound-healing process (Schaffer et al 1996). The inflammatory response associated with wound healing points towards iNOS as the favoured isoform to be tested as a gene therapy treatment. iNOS gene expression is maximal 24 h after wounding, with a steady decline in expression up to 10 days after transfection (Lee et al 2001). Thornton et al (1998) successfully over-expressed the iNOS gene in cutaneous wounds in rats (Thornton et al 1998). Using only naked iNOS DNA, $NO^{\cdot}$ production was sufficient to increase collagen levels within the wound, thus promoting healing (Thornton et al 1998). Yamasaki et al (1998) measured the rate of wound closure in iNOS –/– mice following a 30 min topical administration of AdV–iNOS, while AdV-LacZ was administered as a control (Yamasaki et al 1998). Wound closure took a mean of 27.3 days in iNOS–/– mice, compared with 17 days in wild-type controls. One topical application of this iNOS gene therapy was enough to significantly improve wound healing. This treatment could therefore have clinical applications for aging, patients with diabetes and those taking regular doses of corticosteroids.

iNOS gene therapy for cancer

$NO^{\cdot}$ generation using donor drugs

The first report of the presence of NOS in a tumour was in 1991, in a human colorectal adenocarcinoma cell line (Radomski et al 1991). Cells from both the primary tumour (SW-480) and from its lymph node metastases (SW-620) were shown to have calcium-independent NOS activity. Furthermore, treatment of the less-metastatic SW-480 cells with nitro-L-arginine (L-NA; an NOS inhibitor) increased their metastatic potential to that of the SW-620 cells (Radomski et al 1991). This led to the first hypothesis that $NO^{\cdot}$ overexpression evoked strong anti-tumour effects.

In relation to tumourigenesis, it is widely accepted that low levels of $NO^{\cdot}$ correlate strongly with pro-tumour effects, whilst high levels result in tumour inhibition (Xie & Fidler 1998; Kim et al 2001; Wang et al 2004). The direct and indirect interactions of $NO^{\cdot}$ influence a plethora of events associated with tumour progression in particular. With regard to angiogenesis, Jones et al (2004) demonstrated the dose-dependent effect of $NO^{\cdot}$ on tubule formation, where treatment with low levels (0.1–0.3 mM) of the $NO^{\cdot}$-donor drug SNAP significantly increased pro-angiogenic effects. However,

increasing the concentration of SNAP above 0.5 mM resulted in an 80% inhibition of tubule formation. Furthermore, the mechanisms whereby $NO^{\cdot}$ can induce chemical damage to DNA to result in apoptosis include the formation of reactive nitrogen oxide species (RNOS), formed from $NO^{\cdot}$ and reactive oxygen species (ROS). These ROS include superoxide (O_2^-), which, when reacted with $NO^{\cdot}$, forms the apoptosis-inducer peroxynitrite ($ONOO^-$) (Kim et al 2001; Porasuphatana et al 2003). Peroxynitrite acts as a highly potent DNA oxidant, which results in the formation of single-strand breaks. This is particularly evident during replication and transcription when DNA is in single-strand form and therefore more susceptible. DNA breakage induces some secondary effects, including accumulation of the tumour suppressor gene p53 and activation of the nuclear enzyme poly-ADP-ribose polymerase (PARP), which are directly involved in the detection and signalling of single-strand breaks, and have important roles in maintaining genome stability (Kim et al 2001). PARP proteins contain two zinc finger motifs which are damaged in the presence of RNOS, losing their integrity, and ultimately resulting in the inhibition of DNA repair (Xu et al 2002b).

The role of the mitochondria in inducing apoptosis also appears to be linked to $NO^{\cdot}$ generation. Expression of $NO^{\cdot}$ induces changes in the mitochondrial permeability transition pore (MPTP). Opening of the MPTP allows the release of cytochrome *c* into the cytoplasm during early apoptosis. This has multiple cascade effects, which include activation of caspases by binding to apoptotic peptidase activating factor 1 (Apaf-1), a structural protein that facilitates activation of caspase 9 in the presence of ATP or deoxyadenosine triphosphate (dATP) (Zou et al 1999). Once activated, caspase 9 cleaves caspases 3 and 7, resulting in their activation and subsequent apoptotic events. Furthermore, the $NO^{\cdot}$-induced release of cytochrome *c* leads to a massive release of intracellular calcium via interactions with inositol triphosphate, resulting in cell death (Sedlak & Snyder 2006). This is illustrated in Figure 2.

Overexpression of endogenous $NO^{\cdot}$

Many of the aforementioned studies rely on the generation of $NO^{\cdot}$ from donor drugs. Although positive anti-cancer effects have been reported, safety concerns remain with regard to translational studies. Treatment of systemic disease with $NO^{\cdot}$-donor drugs raises problems of non-target cell toxicity and systemic hypotension. Gene therapy offers an alternative approach for the specific delivery of $NO^{\cdot}$. In the search for

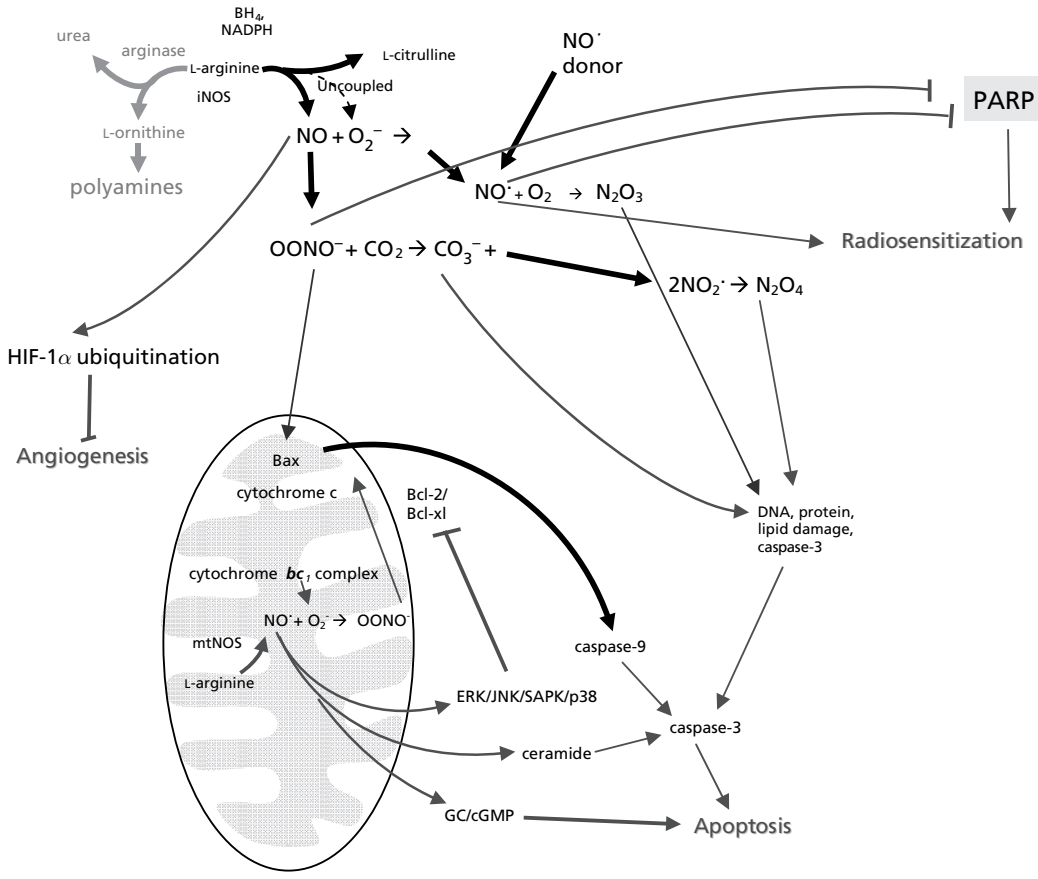

Figure 2 Pathways activated by high concentrations of nitric oxide ($NO^{\cdot}$) for cancer therapeutics. Schematic representation of the various pathways and anti-tumour endpoints elicited through the overexpression of $NO^{\cdot}$. Enhanced therapeutic effects are generated via anti-angiogenic effects to inhibit tumour proliferation. Radiosensitization subsequently increases the therapeutic index of conventional treatment modalities. Apoptosis is induced directly via the cleavage of various caspases.

HIF, hypoxia-inducible factor; $ONOO^-$, peroxynitrite; $NO^{\cdot}$, nitric oxide; PARP, poly-ADP-ribose polymerase

novel cancer therapies, several groups have utilized the properties of the iNOS enzyme in a variety of gene therapy approaches to produce strong anti-tumour effects.

Xie. et al (1995) were one of the first groups to demonstrate the anti-cancer effect of iNOS overexpression. A colony (C4.P) of the murine melanoma K-1735 cell line that is highly metastatic and has low endogenous expression of iNOS was transfected with a functional iNOS gene (C4.L8), an inactivated-muted iNOS gene (C4.S2) or a neomycin-resistance gene (C4.Neo). Following identification of positive transfectants, viable cells were harvested and used to assess the tumourigenic and metastatic potential of the variant cell lines, via i.v. injection into BALB/c nude or C3H/HeN mice. Animals injected with the C4.P, C4.S2 and C4.Neo cells showed extensive metastases, whereas the survival and metastatic potential of K-1735 C4.L8 cells was abrogated 48–72 h after the injection. Furthermore, a daily 20 mg injection of the NOS inhibitor N^G-methyl-L-arginine (L-NMA) increased the survival of all the cell variants, including the C4.L8 variant carrying the functional iNOS gene. This in-vivo experiment clearly demonstrated the cytotoxic effects that could be mediated through overexpression of the iNOS gene (Xie et al 1995). In-vitro analysis of the iNOS-transfected K-1735 cells indicated an elevated apoptotic fraction compared with the control variants (Xie & Fidler 1998). Further investigation demonstrated that toxicity resulting from iNOS overexpression was not restricted to the transfected cell, but that high-level $NO^{\cdot}$ generation induced cytolysis of bystander cells, both in-vitro and in-vivo. In a follow-up study, overexpression of $NO^{\cdot}$ was achieved through multiple i.v. injections of a liposome complex containing a synthetic lipopeptide capable of up-regulating iNOS protein expression. The multilamellar vesicle-liposomes contained the lipopeptide CGP31362, which activates the tumouricidal properties of macrophages and subsequently induces iNOS. This ultimately resulted in complete tumour regression of a murine M5706 reticulum sarcoma model (Xie & Fidler 1998).

Overexpression of transduced $NO^{\cdot}$

Although early studies focused on overexpression of endogenous iNOS, the therapeutic potential of $NO^{\cdot}$ overexpression was evident, forming the basis for future iNOS gene therapy strategies involving the direct delivery of the iNOS gene to tumour cells or other target cells through liposomal or viral vectors.

Viral vector gene therapy strategies Carcinoembryonic antigen (CEA) is one of a class of glycosylated proteins that are widely expressed on the surface of tumour cells of many human tissues, and as such may be a useful target molecule for gene therapy using an anti-CEA antibody (Fichera et al 1998). Khare et al (2001) first focused on the autocytotoxicity induced by $NO^{\cdot}$ overexpression and the cytotoxicity induced in surrounding bystander cells. They developed a gene therapy strategy, delivering the iNOS gene in a retrovirus vector displaying a single-chain variable fragmented antibody to CEA. A modified chimeric antibody (Ch F11-39) with a high affinity for CEA-expressing tissue was developed from a F11-39 antibody clone. This clone also had the ability to distinguish between normal CEA-expressing tissue and tumour tissue, providing an extra level of specificity (Kuroki et al 1992). RT-PCR analysis of cells infected with variations of the retrovirus-reconstructed packaging cells confirmed iNOS expression as a result of gene therapy treatment. Specificity of expression was confirmed by an in-vitro cytotoxicity assay, where MKN-74 (CAE-negative) cells and MKN-45, KATO-III and CHO-CEA (CAE-positive) cells lines were exposed to the GPEscFv-env-iNOS retrovirus. No significant cytotoxicity was observed following exposure of the CEA-negative cells to the iNOS-expressing retrovirus. This was in stark contrast with the CEA-positive cells, in which the surviving fraction was reduced to 18–25%. The selective uptake of the viral package by CEA-expressing cells therefore provides a means of restricting non-target-cell toxicity (Khare et al 2001).

A series of experiments by Chung et al (2003) demonstrated an increase in the apoptotic fraction of tumour cells infected with an AV vector carrying the human iNOS gene under the control of the CMV promoter. A 4-fold increase in tumour apoptosis was observed following radiation when HCT-116 cells were either transduced with AdV–iNOS gene therapy or treated with an $NO^{\cdot}$-donor drug prior to irradiation. Several groups had previously demonstrated that the induction of apoptosis via $NO^{\cdot}$ generation is a caspase-dependent process (Li et al 2004; Royle et al 2004). This was subsequently confirmed, as $NO^{\cdot}$-induced apoptosis was significantly inhibited when cells were pre-treated with the pan-caspase inhibitor Z-VAD-(OME)-FMK. As with other aspects of tumour biology, the effect of $NO^{\cdot}$ in response to p53 status appears to be controversial. Early studies suggested that mutant p53 cells, which express iNOS, were more likely to display pro-tumour characteristics (Ambs et al 1998). In contrast to this theory, Chung et al (2003) suggested that increased radiosensitization is not linked directly to p53 status, as both HCT-116 wild-type p53 and SNU-1040 mutant p53 cells exhibited an increase in apoptosis caused by overexpression of the iNOS gene (Chung et al 2003).

Further research into the combined effects of iNOS overexpression and radiation has revealed a synergistic apoptotic outcome via activation of p53. SNU-1040 cells carry a missense point mutation (TCC→TTC) within the p53 gene, localized to codon 241 of exon 7. This mutation renders the p53 gene non-functional, limiting activation of $p21^{WAF-1}$ downstream, and apoptosis. Cook et al (2004) demonstrated that delivery of a functional copy of the p53 gene using an AdV vector was capable of inducing $p21^{WAF-1}$ protein expression and subsequent apoptosis in 80–90% of cells by 48 h after transfection (Cook et al 2004). Western blot analysis of the AdV–p53 transfected cells confirmed that p53 and $p21^{WAF-1}$ proteins were present in the transfected cells but absent from the untransfected cells. The activation of p53 was further enhanced in the presence of iNOS-generated $NO^{\cdot}$. This mechanism of action was hypothesized to be through phosphorylation of p53 at serine 15. In-vivo, HCT-116 xenograft tumours were treated for 2 consecutive days with direct injections of the AdV–iNOS gene therapy, AdV–CTL (non-toxic, sham vector) or no treatment, followed by a single 2 Gy radiation dose on the third day. Tumours treated with the AdV–iNOS plus radiation exhibited highly significant increases in serine-15 phosphorylation, as well as a 55-fold

increase in the number of cells expressing the active form of p53, compared with all other treatment combinations (Cook et al 2004).

Non-viral vector gene therapy strategies While high levels of transgene expression can be obtained through viral-based gene therapy approaches, many groups continue to develop non-viral delivery systems in attempts to eliminate the potential of evoking an adverse immune response (Lehrman 1999). The highly irregular nature of tumour vasculature leads to the development of regions of persistent hypoxia, causing resistance to both chemotherapy and radiotherapy. The heterogeneous perfusion of tumour tissue also limits the effective delivery of chemotherapeutic agents, as well as substantially reducing oxygen tension and subsequently increasing radioresistance (Tatum et al 2006).

Our group first aimed to utilize the iNOS gene to manipulate the tumour microenvironment through the potent vasodilator properties of $NO^{\cdot}$ (Worthington et al 2000). To add specificity to our gene therapy plasmid, we controlled expression using the wild-type p53-activated fragment 1 (WAF-1, also known as p21 or Cip-1) promoter. Following a series of proof-of-principle experiments using a green fluorescent protein (GFP) reporter gene construct, we determined that 4 Gy was the optimal priming dose required for maximal transgene activation. Ex-vivo preparations of rat tail artery were cannulated and attached to the perfusion apparatus. The sections were exposed to the vasoconstrictor phenylephrine and normal responses recorded. Sections were then transfected for a 2 h period using a cationic lipid transfection reagent (Tfx-50). Immediately after irradiation, normal responses to phenylephrine were observed. However, within 60 min after transfection a 65% decrease in the contraction response was observed. This inhibition of contraction was due to $NO^{\cdot}$ overexpression from the transfected iNOS gene, as treatment with the specific iNOS inhibitor, nitro-L-arginine returned contraction to 75% of untransfected sections. Western blot analysis confirmed highly significant increases in iNOS protein levels in rat tail artery sections transfected with the WAF-1–iNOS plasmid compared with non-transfected controls (Worthington et al 2000). By promoting specific vasodilatation in this approach, tumour oxygenation levels may be enhanced, allowing a greater level of cell kill by subsequent therapeutic treatments.

In a follow-up to our ex-vivo WAF-1–iNOS gene therapy study, we assessed the anti-cancer potential of iNOS constructs under the control of a constitutive viral promoter (CMV) and the WAF-1 radiation-inducible promoter. This novel gene therapy strategy delivered the iNOS gene using cationic liposomal vectors. RIF-1 murine fibrosarcoma cells were used to illustrate an increase in iNOS protein expression following transient transfection of the iNOS gene. This overexpression in turn resulted in elevated intracellular $NO^{\cdot}$, causing radiosensitization of hypoxic tumour cells in-vitro, and delay of tumour growth in-vivo. Variations in endogenous iNOS protein levels were observed between hypoxic- and normoxic-maintained RIF-1 cells; transfection with the iNOS transgene resulted in 2.9- and 5.9-fold increases in iNOS protein expression, respectively. Radiosensitization following gene therapy was assessed by clonogenic assay. RIF-1 cells maintained under normoxic conditions and exposed to radiation were approximately 3-fold more sensitive to radiation than cells maintained in $95\%N_2/5\%CO_2$ for 1 h. However, following transfection with the iNOS constructs, radioresistance observed under hypoxia was effectively eliminated. Further confirmation of the radiosensitization properties of $NO^{\cdot}$ was obtained following treatment with the NOS inhibitor L-N^G-monomethyl arginine (L-NMMA), which eliminated radiosensitization by iNOS transfection in hypoxic cells. In an in-vivo setting, the combination of a 20 Gy radiation dose and CMV–iNOS gene therapy resulted in a highly significant delay in tumour quadrupling time, to 19.7 days for the combination therapy compared with 2.9 days for a 20 Gy radiation dose alone (Worthington et al 2002). This was the first non-viral gene therapy study to specifically target tumour cells with the iNOS gene and second only to that of the retrovirus study by Khare et al (2001) targeting tumour cell CEA expression.

Xu et al (2002a) describe an elegant series of experiments where human fetal kidney cells (EcR293) were transfected with human iNOS cDNA before being microencapsulated within a semipermeable alginate-poly-L-lysine membrane. These microencapsulated cells could then be fully induced to produce high levels of $NO^{\cdot}$ in the presence of ponasterone A. Therapeutic efficacy of the model was determined using a xenograft nude mouse model in which either a DLD-1 human colon adenocarcinoma or SKOV-3 human ovarian carcinoma were implanted. Over a 28-day period the volume of DLD-1 tumours decreased by 54% compared with control tumours not exposed to the induced microencapsulated EcR293 cells. These effects were further augmented in the ovarian carcinoma model, with almost complete inhibition of tumourigenic potential. This was the first study to demonstrate that iNOS-directed cell killing is associated with an upregulation of Fas/FasL, which subsequently activate apoptosis-inducing pathways.

Worthington et al (2005) developed a non-viral iNOS gene therapy strategy, utilizing the properties of a synthetic radiation-inducible promoter, pE9. This synthetic promoter was developed from a series of CArG repeats isolated from the Egr1 gene, which was shown to upregulate gene transcription following exposure to ionizing radiation (Datta et al 1993). This promoter has been used extensively in the development of alternative gene therapy strategies in combination with the HSV-*tk* gene, with the aim of sensitizing glioma cells to the effects of ganciclovir following a single 3 Gy dose of radiation (Marples et al 2000, 2002). Using this promoter to drive the iNOS transgene, an 8-fold increase in iNOS protein expression was observed compared with cells exposed to the lipofectin reagent alone (Worthington et al 2005). Furthermore, iNOS protein expression under control of the pE9 promoter was comparable to that achieved following transfection of the iNOS gene under the control of the strong CMV viral promoter. The addition of the NOS inhibitor L-NMMA to the cells at the time of transfection significantly abrogated the expression of iNOS protein, to the levels observed in cells exposed to the transfection reagent alone. The anti-tumour potential of the pE9–iNOS construct was also assessed in-vivo following a monotherapy regimen. Established RIF-1 tumours of 150–200 mm^3 were directly injected with 25 μg of

empty vector, CMV–iNOS or pE9–iNOS, and efficacy was determined by the time taken for tumours to reach 3 times their volume on the day of treatment. Untreated control tumours reached 3 times the initial tumour volume within 4.2 days, and 5.1 days following treatment with the empty vector. Direct injections of pE9–iNOS resulted in highly significant growth delays, which equated to a further 6.9- and 6.0-day growth delay compared with untreated controls and vector-only-treated tumours, respectively. Further investigation of the tumours treated with the pE9–iNOS constructs revealed enhanced iNOS protein expression, cumulating in significantly increased intra-tumour nitrite levels compared with tumours treated with the trans-IT empty vector (Worthington et al 2005). Again, this approach clearly demonstrates the cytotoxic potential of $NO^{\cdot}$ overexpression, resulting in the highly significant tumour growth delays achieved in the absence of any inducing radiation dose, although, like many other strategies, it lacks tumour-cell specificity.

Potentially the most useful gene therapy strategies will augment the therapeutic potential of current protocols, without requiring significant alterations to those regimens. McCarthy et al (2007a) developed a fractionated radiation regimen to mimic that of current radiotherapy modalities, and combined it with delivery of the iNOS transgene under the control of the radiation-inducible $p21^{WAF-1}$ promoter. This study extended previous work which had demonstrated highly selective transcriptional targeting using the $p21^{WAF-1}$ promoter, as well as radiosensitization of tumour cells in-vitro and delayed tumour regrowth in-vivo, using large doses of X-rays (Worthington et al 2002, 2004). Various cell lines of differing p53 status were transfected with the WAF-1–iNOS construct, and exposed to 2 Gy incremental X-rays doses, to a maximum dose of 6 Gy. Interestingly, reporter gene activation was most pronounced in DU145 and HT29 cell lines, which lacked functional p53. This corroborated existing evidence supporting $p21^{WAF-1}$ activation independent of p53 status and suggests an independent role for $p21^{WAF-1}$ function in relation to radiation therapy (Akashi et al 1995; Gartel & Tyner 1999; Wouters et al 1999). Additionally, in-vivo treatment with the WAF-1–iNOS construct in combination with fractionated radiotherapy generated highly significant reductions in tumour growth. RIF-1 and HT29 intradermal tumour models were established and animals assigned to a variety of treatment groups based on a combination of gene therapy, initial radiotherapy priming dose and therapeutic fractionated doses of 2 Gy. In both models, a 50% increase was observed in the time taken for growth to reach termination point following monotherapy with the WAF-1–iNOS gene construct and fractionated 2 Gy doses of radiation compared with tumours treated with a vector-only construct.

An alternative to exogenously activated promoters such as $p21^{WAF-1}$ and pE9 is a class of promoters that are tissue or tumour-type specific. Activation of these promoter sequences is generally through various locally upregulated transcription factors, conferring the required level of target cell specificity. The full potential of these promoters has yet to be realized, however, as high specificity has generally come at the price of weak transgene expression (Robson & Hirst 2003).

The development of prostate cancer into an androgen-independent state is indicative of poor tumour responses to current treatment modalities, commonly resulting in the development of secondary metastatic bone lesions (Feldman & Feldman 2001; Petrylak 2002). The precise mechanism resulting in the enhanced osteomimetic nature of hormone-refractory prostate is not fully understood, although upregulation of bone-like proteins such as bone sialoprotein, osteopontin and osteocalcin (OC) is commonly observed. The promoter activity of these genes is also enhanced and, in particular, transcription factors such as RunX-2, Fra-2 and Jun-D have been shown to be responsible for the activity of the human OC (hOC) promoter (Curatolo et al 1992; Dodds et al 1995; Koeneman et al 1999, 2000). McCarthy et al (2007b) have recently cloned the hOC promoter to drive the iNOS gene, specifically targeting hormone-refractory prostate cancer. Promoter specificity was determined using a GFP reporter gene construct, where GFP expression was shown to be restricted to PC3 and DU145 androgen-independent prostate cancer cell lines. Furthermore, specificity was maintained when the cells were transfected with the hOC–iNOS construct, resulting in enhanced iNOS protein expression and elevated nitrite concentrations (Table 2) in the tissue culture media.

The anti-tumour effects of the constructs were determined in-vitro using the well-established clonogenic assay. In the presence of BH_4, a necessary and often rate-limiting co-factor for NOS activity, the clonogenicity of PC3 and DU145 cells was reduced by 78% and 73%, respectively (McCarthy et al 2007b). Furthermore, the hOC–iNOS gene construct generated cytotoxic effects comparable to those obtained following transfection with CMV–iNOS, alleviating the concerns relating to specificity and strength of promoter activation. These promising findings provide a platform for future in-vivo studies and the possible development of treatments that provide more than a palliative outcome.

Limitations of clinical application

Despite the potential gene therapy strategies that have progressed to phase I, II and III clinical trials, and the numerous preclinical protocols, gene therapy has not proved to be anywhere near as successful as first predicted. This may be due in part to low and transient transfection efficiencies and transgene expression (Tait et al 1997; Hesdorffer et al 1998; Knop et al 1999). Furthermore, concerns regarding the safety of these potential treatments remain a major stumbling block. This was particularly evident following the death of a patient 4 days into a series of treatments for ornithine transcarbamylase deficiency, where the body's ability to rid itself of ammonia is severely inhibited. In addition, several specific issues have limited the clinical development of iNOS-based gene therapy strategies. The primary concern is directly linked to the specificity of expression. If expression is not tightly regulated to the target site, systemic hypotension is a real concern, effectively mimicking the effects of endotoxic shock (Wang et al 1999; Halliwill et al 2000). Although thousands of patients have been treated with gene transfer therapies, reproducibility of positive results has yet to be demonstrated conclusively, meaning that gene therapy as an everyday treatment is still some way off. However, it is important to keep in mind the relatively

Table 2 Mean increase of total nitrite concentration in a range of tumour cell lines following inducible nitric oxide synthase (iNOS) gene therapy under the control of the cytomegalovirus (CMV) and human osteocalcin (hOC) promoters

Cell line	Treatment group	Total nitrite concn (μM) (mean ± s.e.)	Gene-therapy-induced nitrite concn (μM)
PC-3	Control	8.4 (1.62)	
	CMV/iNOS	13.65 (1.88)	4.71
	hOC/iNOS	13.79 (2.56)	4.85
DU145	Control	9 (2.02)	
	CMV/iNOS	15.08 (3.78)	6.08
	hOC/iNOS	14.78 (4.05)	5.78
LNCaP	Control	7.19 (0.267)	
	CMV/iNOS	13.71 (0.33)	6.52
	hOC/iNOS	7.75 (0.175)	0.56
HT29	Control	5.62 (0.541)	
	CMV/iNOS	10.17 (0.75)	4.55
	hOC/iNOS	6.01 (0.88)	0.39
MCF-7	Control	8.41 (1.15)	
	CMV/iNOS	14.09 (0.75)	5.68
	hOC/iNOS	14.16 (0.68)	5.75
RIF-1	Control	8.06 (0.13)	
	CMV/iNOS	13.52 (0.50)	5.46
	hOC/iNOS	13.07 (0.63)	5.01

short period of time taken for these theories to become practice in clinical trials.

Conclusion

Vascular diseases are associated with reduced $NO^{\cdot}$ bioavailability; thus, NOS gene therapy is an ideal candidate for treatment of such pathologies. iNOS gene transfer has advantages over the transfer of other NOS isoforms or $NO^{\cdot}$-donor drugs. Compared with eNOS and nNOS, iNOS synthesizes much higher quantities of $NO^{\cdot}$ and is not dependent on intracellular calcium fluxes, resulting in consistent $NO^{\cdot}$ production within tissue (Kelly et al 1996). Even with low transduction efficiency, highly significant responses have been observed. In one study, 18% of cells expressing iNOS resulted in a 67% infarct reduction (Tzeng et al 1996; Li et al 2003). Indeed, AdV–iNOS overexpression has been shown in many studies to elicit substantial cardioprotective properties. Furthermore, iNOS gene therapy presents a clinically viable treatment because it can be delivered locally to the vascular lesion through balloon-coated catheters, direct catheters, stents or aerosolization. This eliminates many of the toxicity concerns associated with systemic delivery.

In the search for new oncotherapies, many groups have harnessed the potent anti-cancer nature of $NO^{\cdot}$ when developing gene therapy strategies utilizing iNOS. Unlike many of the cardiac pathologies, where controversy surrounds the most appropriate isoform of the NOS enzyme for treatment purposes, there is no ambiguity regarding the treatment of cancer. Low levels of $NO^{\cdot}$ generation are more commonly associated with the expression of eNOS and nNOS. These are strongly linked to tumour progression through a myriad of pro-tumour events such as increased DNA mutagenesis, angiogenesis and invasiveness, whilst inhibiting tumour cell apoptosis (Hajri et al 1998; Xie & Huang 2003). Conversely, micromolar concentrations of $NO^{\cdot}$ generated by the expression of iNOS are associated with increased DNA damage, oxidative/nitrosative stress, induction of apoptosis and mitochondrial damage (Mocellin et al 2007). The aforementioned studies have all outlined several gene therapy strategies, both in-vitro and in-vivo, highlighting the potential of iNOS gene therapy for the treatment of a range of tumour types. Future developments should focus on targeted systemic delivery of the gene, with the aim of validating its efficacy in the clinical setting.

It is now 20 years since $NO^{\cdot}$ was first widely recognized as being more than an insignificant curiosity. We now know that it plays vital roles in all tissues, and offers the potential for novel therapies to treat a wide range of pathologies.

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