AD_____

Award Number: DAMD17-98-1-8495

TITLE: Nitric Oxide Gene Therapy for Prostate Cancer

PRINCIPAL INVESTIGATOR: Elwood Armour, Ph.D.

CONTRACTING ORGANIZATION: William Beaumont Hospital
Royal Oak, Michigan 48073-6769

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (01 Oct 98 - 30 Sep 99)

4. TITLE AND SUBTITLE
Nitric Oxide Gene Therapy for Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8495

6. AUTHOR(S)
Elwood Armour, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
William Beaumont Hospital
Royal Oak, Michigan 48073-6769

e-mail:
warmour@beaumont.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Traditional therapies for advanced prostate cancer are unable to cure a majority of patients. One approach to therapy is over-production of inducible nitric oxide synthase (iNOS) within the tumor by injecting replication defective adenovirus containing the DNA sequences for iNOS into prostate tumors. The adenovirus gene transfer technology is readily applicable and an iNOS, with CMV promoter, containing adenovirus (Ad5-CMV-iNOS) has been constructed and tested in our laboratory. The production of nitric oxide in DU-145 prostate cancer cells growing *in vitro* after infection with Ad5-CMV-iNOS has been determine. Maximum NO release rate occurred when cells were infected at multiplicity of infection (MOI) 15 and after 24 to 36 hours incubation. Direct cell killing commenced within 3 days after infection and occurred at MOI greater than 5. In the second phase of the project DU-145 and PC-3 cells will be grown as xenograft tumors in nude mice, infected by injection with Ad5-CMV-iNOS, and analyzed for nitric oxide, superoxide, peroxynitrite, and hydrogen peroxide production, cytotoxicity, and radiation sensitization. These results will help determine the best approach to applying iNOS gene therapy to the treatment of prostate cancer in human patients.

14. SUBJECT TERMS
Prostate

15. NUMBER OF PAGES
22

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Elwood P Armour Oct 26, 1999

PI - Signature Date

Table of Contents

(5) INTRODUCTION

Traditional therapies for advanced prostate cancer are unable to cure a majority of patients. Improving local control of the primary tumor is a practical means of increasing the cure rate. Local failure after radiation therapy may result from treatment resistant hypoxic cells. Preferentially killing or radiation sensitizing hypoxic and/or nutrient deprived cells should improve local response and cure rate. One approach to cytotoxic and sensitization therapy is over-production of inducible nitric oxide synthase (iNOS) within the tumor. We propose to accomplish this goal by injecting recombinant replication defective adenovirus containing the DNA sequences for expression of iNOS into prostate tumors. The adenovirus gene transfer technology is readily applicable and an iNOS, with CMV promoter, containing adenovirus (Ad5-CMV-iNOS) has been constructed and tested in our laboratory. The technology to inject virus suspension into prostate tumors is readily available. Currently used high dose rate afterloading (HDR) brachytherapy for prostate tumors could be adapted to include ultrasound guided virus injections. The currently proposed project will determine the chemical and biological responses of Ad5-CMV-iNOS infected prostate cancer cells growing *in vitro* or as tumors in nude mice. The first phase of this project will determine the types and quantities of toxic chemical species produced in DU-145 and PC-3 prostate cancer cells in monolayer culture or as multicell spheroids. Emphasis will be placed on determining changes in iNOS generation of nitric oxide, superoxide, peroxynitrite, and hydrogen peroxide at low concentrations of oxygen and arginine. Direct cell killing and radiation sensitization will also be determined in Ad5-CMV-iNOS infected cells. In the second phase of the project DU-145 and PC-3 cells will be grown as xenograft tumors in nude mice, infected by injection with Ad5-CMV-iNOS, and analyzed for nitric oxide, superoxide, peroxynitrite, and hydrogen peroxide production, cytotoxicity, and radiation sensitization. These results will help determine the best approach to applying iNOS gene therapy to the treatment of prostate cancer in human patients.

(6) BODY

Nitric Oxide Gene Therapy for Prostate Cancer

Task I.....Determine O_2 and arginine dependency of iNOS catalyzed products in human prostate tumor cells

A. Analyze the generation of NO, $O^{\bullet -}_2$, H_2O_2, and $ONOO^-$ by Ad5-CMV-iNOS infection of DU-145 and PC-3 cells at various O_2 concentrations. The microelectrodes, Griess assay, luminescent and HPLC will be used for analysis.
B. Analysis as described in "A" except that arginine concentration is varied.

Experiments were performed in which nitric oxide was measured in DU-145 cells exposed to iNOS-Adenovirus at defined MOI and for various durations of expression.

Nitric oxide microsensors were prepared as described previously. Briefly, a single sharpened carbon fiber (0.5 to 7.0 μm diameter) encapsulated in a glass capillary with 1 mm protruding (for the extracellular measurements) was modified by coating with nickel(II) tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin by use of cyclic scanning at a potential between -0.2 and 1.0 V. The polymeric porphyrin was subsequently coated by dipping for 5 s in 1% Nation-solution and left to dry in air. Linear calibration curves were constructed for each sensor from $2*10^{-9}$ to $2*10^{-5}$ mol/L NO, before and after measurements, with aliquots of saturated NO standard. The porphyrinic microsensor has a response time of 0.1 ms at micromolar NO concentrations and 10 ms at the detection limit of 1 nmol/L. Platinum wire counter electrode and a silver/silver chloride electrode (SSCE) for reference are placed in contact with measurement media.

For the nitric oxide expression experiments, cells were seeded into 35 mm tissue culture dishes at a concentration of 100,000 cells per dish 24 hours prior to infection. At the designated time after infection, culture medium was changed for serum free medium without L-arginine. Cells were then incubated at 37°C for 1 hour in the L-arginine free medium.

Chronoamperometeric measurements (CA) were performed using a PAR model 273 voltammetric analyzer (potentiostat + waveform generator) interfaced with an Pentium II 450 MHz computer with data acquisition and control software. CA, fixed at the peak potential for the oxidation of NO vs SSCE, was used for continuous measurement of the changes of NO concentration from its basal level with time. Because the sensor is located on the surface of cells, and only a very small volume (10 μL) for NO release activation is sampled, the concentration of NO measured is a local or surface concentration (not a bulk or global concentration). Nitric oxide release was activated by addition of L-arginine to a final concentration of 1 mmol/L in the medium. Results were expressed as nmol/L nitric oxide released after agonist added.

The cells were placed in the controlled environment microscope stage and the nitric oxide electrode moved to near contact with a group of cells. Baseline current was recorded and arginine was added into the culture dish medium to a concentration of 1 mM. Probe measurement was then continued. Arginine stimulation of nitric oxide production was recorded. The first set of experiments was performed to determine the role of time after infection and MOI on nitric oxide release. Viral vector infection was with either iNOS-AD5 clone 7.1 or as a control, β-Gal-AD5. The MOI used were 15 and 75. The results of these experiments are presented in Figures I-1 and I-2. The iNOS-AD5 produced 295 nmol/L NO when virus was at an MOI of 15 and the incubation

time at 37°C after infection was 24 hours. The background release rate for non-virus infected control DU-145 cells was 52 nmol/L. Increasing the MOI to 75 for the 24 h time point actually produced a lower NO release of 255 nmol/L. Increasing the incubation time to 48 hours resulted in lower release rates for both 15 and 75 MOI. The rates were 170 and 130 nmol/L respectively. After 72 hours post infection incubation at 37°C the nitric oxide release was similar to that at 48 hours, 150 nmol/L for 15 MOI and 130 nmol/L for 75 MOI. The β-Gal AD5 control vector infected cells actually showed a small amount of stimulation of nitric oxide release, probably due to stimulation of endogenous iNOS. The 24 hour post infection incubation time period had nitric oxide release values of 105 nmol/L and 90 nmol/L for 15 and 75 MOI respectively. The values at 48 hours were 65 nmol/L and 70 nmol/L for 15 and 75 MOI respectively. The values at 72 hours were 80 nmol/L and 85 nmol/L for 15 and 75 MOI respectively. For the iNOS-AD5 virus infected cells substantial cellular toxicity was observed as floating and disintegrating cells. The amount of toxicity increased with both MOI and time of incubation after virus infection.

Figure I-1

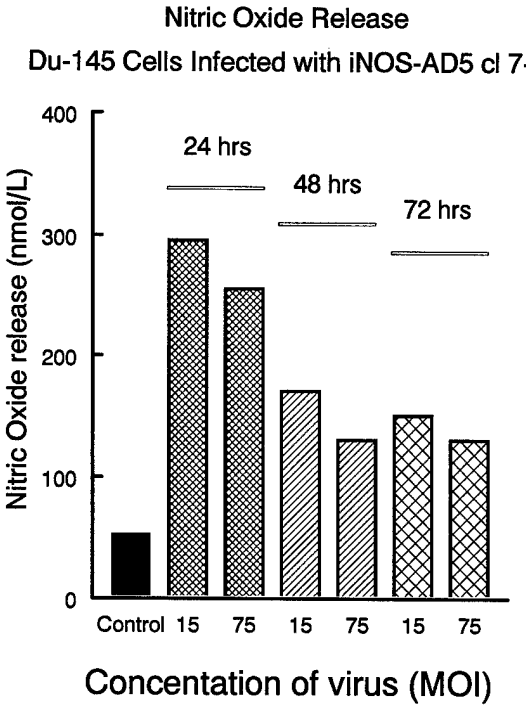
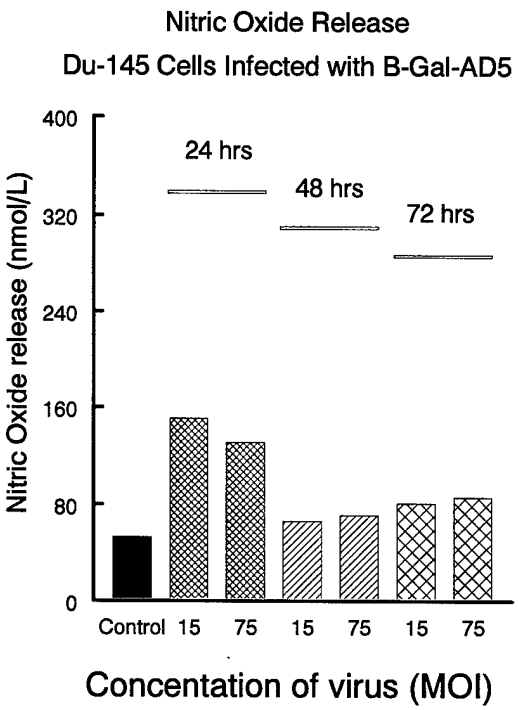
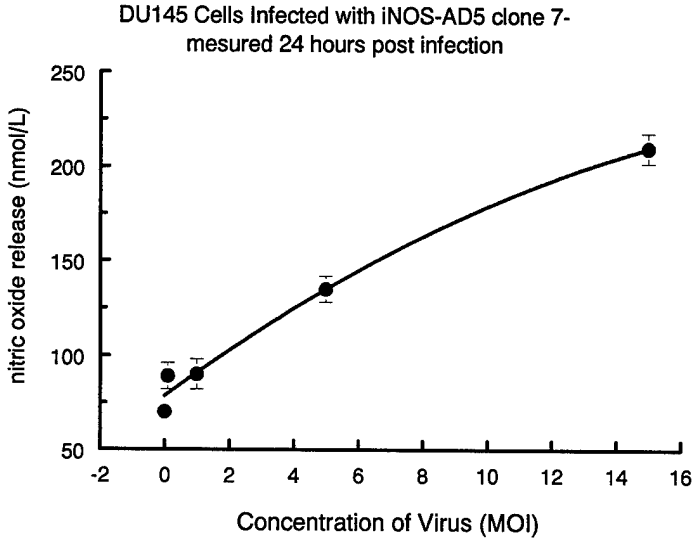


Figure I-2



The next experiments were performed to determine the rate of nitric oxide release at lower MOI of iNOS-AD5 clone 7.1. Because MOI above 15 had produced lower nitric oxide release than that observed at MOI 15, we sought to determine if 15 actually was the peak or if going lower would produce even higher MOI. Cells were grown and infected as described above. The MOI's of iNOS-AD5 used were 0, 0.1, 1, 5, and 15. The results are presented in Figure I-3. The nitric oxide release rate increased with increasing MOI although the curve was flattening at MOI of 15. The release rates for the various MOI were 70 ± 5 nmol/L for MOI 0, 89 ± 7 nmol/L for MOI 0.1, 90 ± 8 nmol/L for MOI 0.5, 135 ± 7 nmol/L for MOI 5, and 210 ± 8 nmol/L for MOI 15.

Figure I-3



The third set of experiments analyzed the role of 37°C incubation time after infection using a wider range of incubation times, especially in the sub 24 hour range. A graphic describing of the experimental protocol is presented in Figure I-4. The results of nitric oxide release rate measurements were presented in Figure I-5. For vector iNOS-AD5 clone 7.1 using MOI 15 the incubation times were 6, 12, 24, 36, and 48 hours. The nitric oxide release rate values were 70 ± 4 nmol/L after 6 hours, 85 ± 3 nmol/L after 12 hours, 152 ± 6 nmol/L after 24 hours, 180 ± 10 nmol/L after 36 hours, and 95 ± 5 nmol/L for 48 hours. A 24 hour post infection point using MOI 30 was also included. The nitric oxide release rate was 50 ± 3 nmol/L for this condition. Another viral vector was also tested. This vector is an AD5 viral clone containing a cDNA sequence of mouse iNOS and the CMV promoter. This vector has been recently developed as described in the next section. The nitric oxide release rate with a 24 hour 37°C incubation after infection for this clone was 190 ± 10 nmol/L.

Figure I-4

iNOS19991001

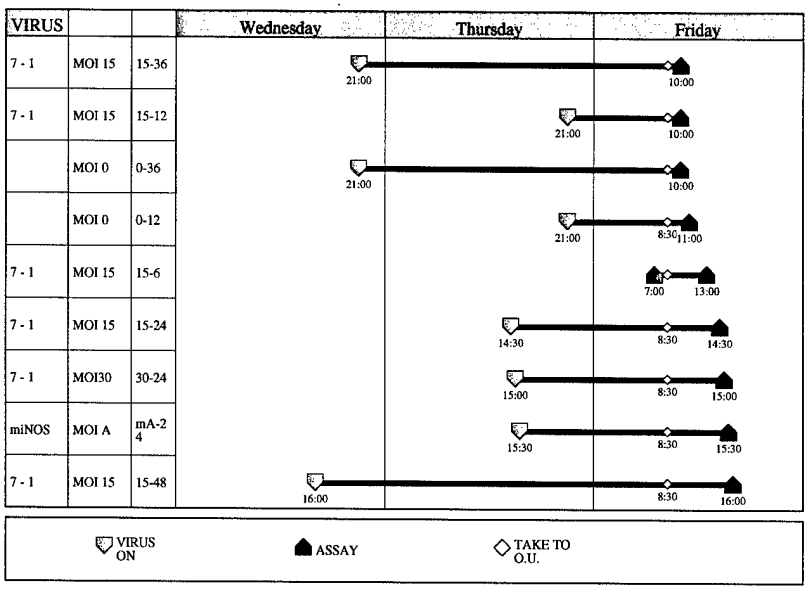
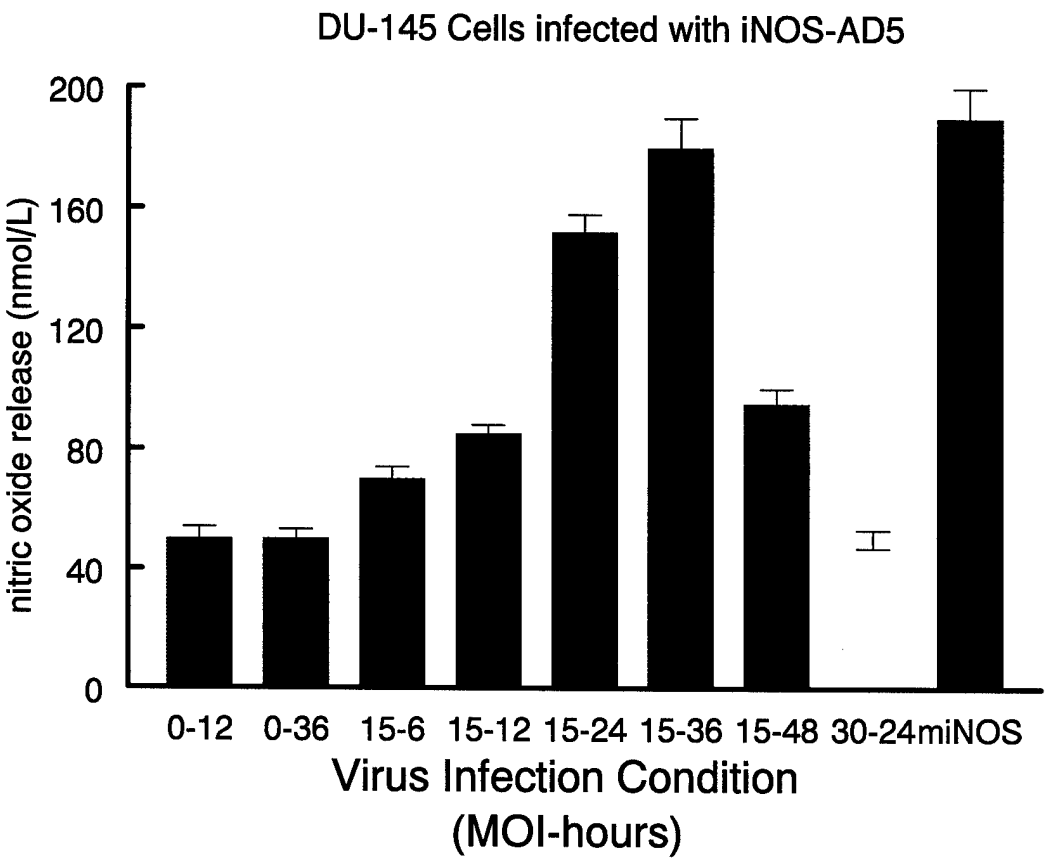


Figure I-5



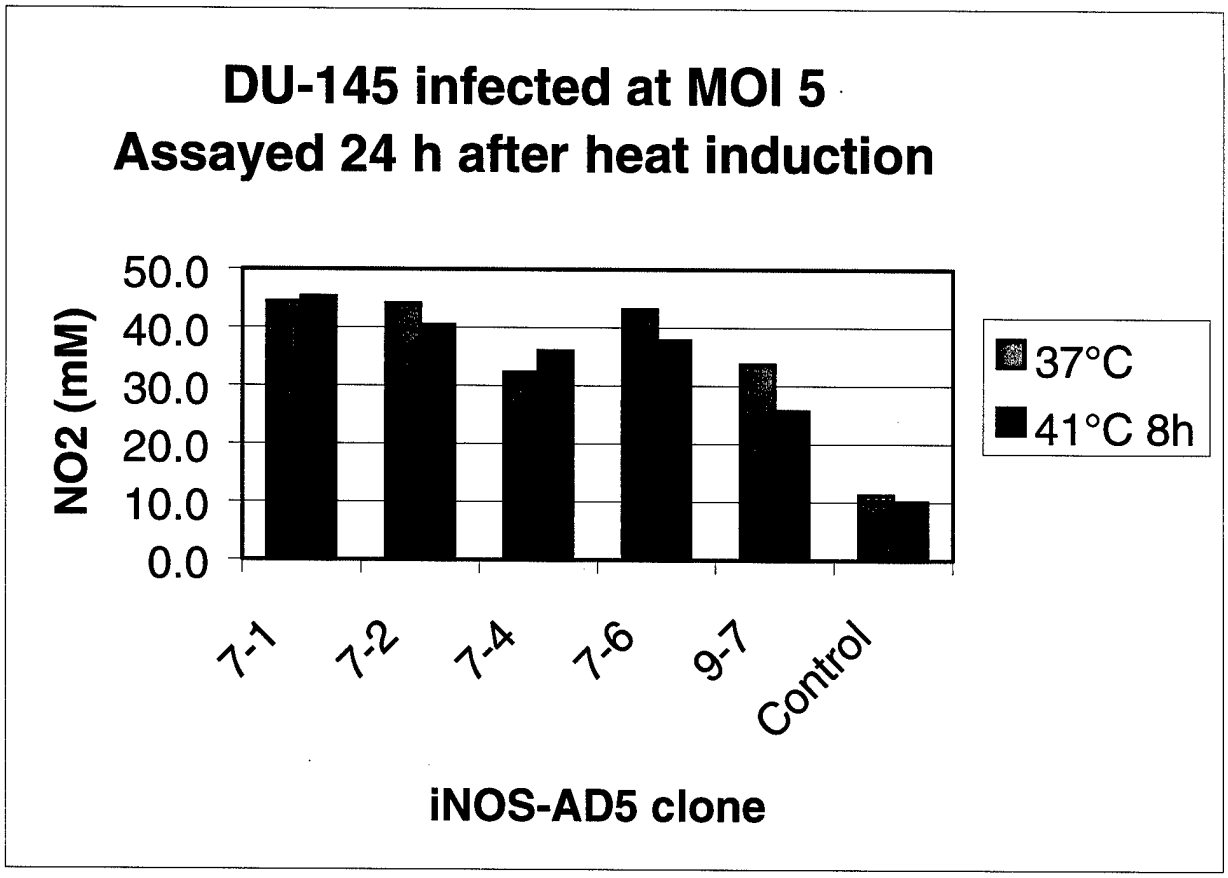
These experiments have demonstrated that the iNOS-AD5 clone 7.1 vector produces a significant amount of nitric oxide in DU-145 cells and that the time course and MOI must be controlled to result in optimal nitric oxide release. Nitric oxide release increased with increasing MOI up to 15 where after a decrease was observed. The nitric oxide release rate also depended upon time of incubation after infection. The maximum release rate was observed at 24 to 36 hours after infection. Combined with the observed cellular toxicity results we conclude that the decrease in nitric oxide release rate above MOI 15 and time of 36 hours is due to loss of macromolecular synthesis and general cellular metabolism as the cells die. The results also indicate that the new mouse iNOS vector miNOS-AD5 produces NO at least as well as the 7-1 iNOS-AD5 clone.

Do our current the "iNOS-AD5" clones have a CMV promoter that is heat inducible?

We learned from our colleague, Dr. Michael Borrelli, that many AD5 virus vectors with a CMV promoter expressed their associated transgene at a much greater rate when infected cells were given a heat shock. The possible benefits of enhancing iNOS expression and thus cell toxicity effects by means of this option interested us enough to do a set of experiments to test whether the "iNOS-AD5" clones have a CMV promoter that is heat inducible. The fact that there are several different forms of the CMV promoter and each behaves differently under various conditions meant that the experimental outcome was uncertain.

DU-145 Cells were incubated in tissue culture dishes for 24 hours prior to infection with iNOS-AD5 viral vector. Five different clones of iNOS-AD5 were used. Several clones were tested because of the possibility of variation in heat enhancement with different clones. Cells were infected at an MOI of 5. After the dishes of cells had incubated at 37°C for twenty four hours after infection, individual dishes were either heat shocked at 41°C for 8 hours or incubated at normal temperature of 37°C for the same length of time. After an additional 16 hours at 37°C an aliquot of medium was removed from the dishes. The medium was assayed for NO2 + NO3 by means of the Griess assay with cadmium conversion of NO3 to NO2. The results presented in Figure I-6 demonstrated that there was no enhancement of NO2 accumulation after the heat shock. The accumulation of NO2 + NO3 in all iNOS-AD5 infected cells was significantly above the control level but no differences between heated and non-heated were observed in any of the five clones.

Figure I-6



Task II.....Determine direct cytotoxicity and radiosensitization resulting from infection of DU-145 and PC-3 cells with Ad5-CMV-iNOS *in vitro*

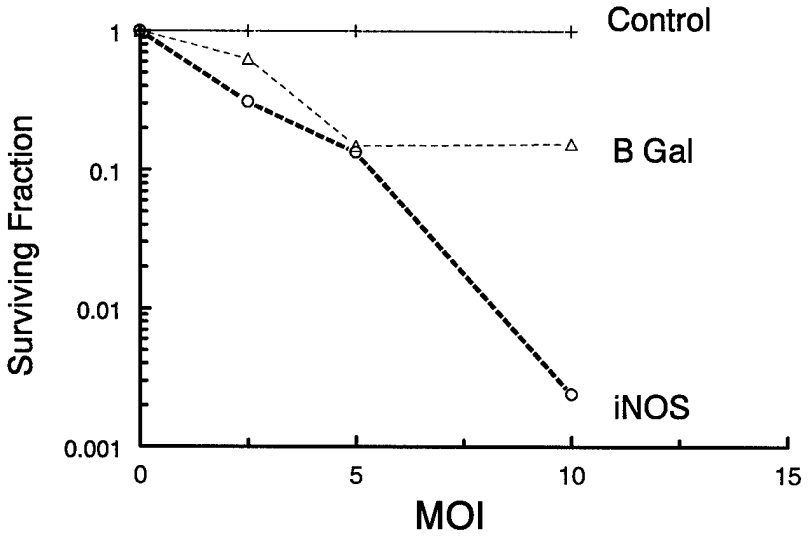
A. Determine direct cytotoxicity of Ad5-CMV-iNOS infection on prostate tumor cells growing in monolayer culture. Colony formation assay will be employed.
B. Determine radiation sensitization of Ad5-CMV-iNOS infection on prostate tumor cells growing in monolayer culture.
C. Determine the role of cell to cell contact and 3 dimensional environment on direct cytotoxicity of Ad5-CMV-iNOS infection on prostate tumor cells growing in multicell spheroids.
D. Determine the role of cell to cell contact and 3 dimensional environment on radiation sensitization of Ad5-CMV-iNOS infection on prostate tumor cells growing in multicell spheroids.

The growth and viability of DU-145 and PC 3 cells was measure after infection with iNOS-AD5 virus vector at various MOI.

Surviving fraction of DU-145 cells was measured using the colony formation assay. Cells were incubated in 60 mm tissue culture dishes for 24 hours prior to infection. Dishes were either infected with no virus, β-Gal-AD5 virus, or iNOS-AD5 clone 7.1 virus. The MOI's used were 2.5, 5 or 10. Cells were incubated under these conditions for 72 hours. At that point they were removed from the dishes by trypsinization and reseeded into dishes to form colonies. The colony formation dishes were incubated for 10 days prior to staining and counting of colonies. With increasing MOI the β-Gal-AD5 virus infected cells suffered a small decrease in colony formation. The iNOS-AD5 infected cells had only slightly more toxicity than the β-Gal-AD5 infected cells at 2.5 and 5 MOI but at 10 MOI the decrease in survival was much greater with survival almost 2 orders of magnitude lower. The data is presented in Figure II-1.

Figure II-1

Survival of DU-145 cells

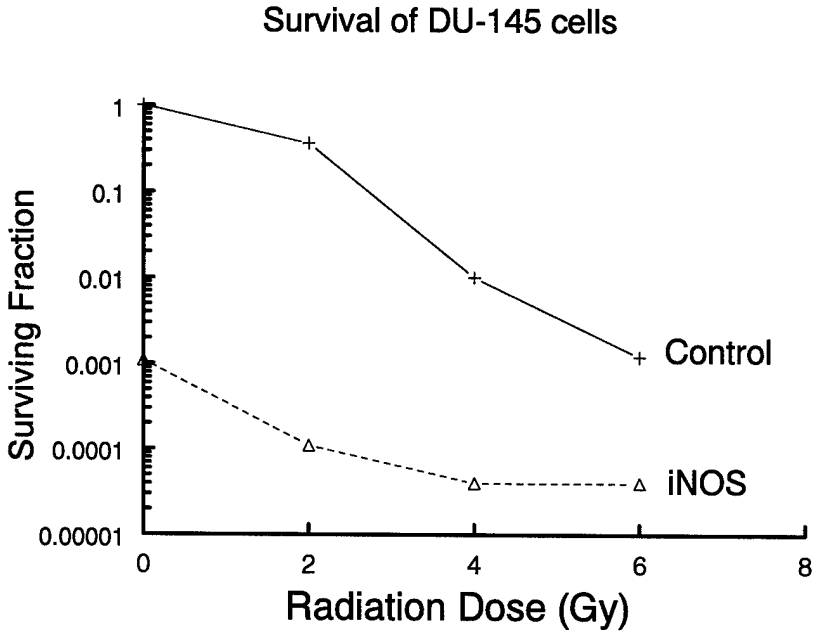


Cells were infected with iNOS-AD5 for 72 hours and seeded for colony formation.

Experiments were performed to measure survival of cells exposed to MOI greater than 10. Survival by colony formation was always less than detectable by colony formation at these higher MOI.

Radiation response survival experiments were performed to determine the survival of cells infected with iNOS-AD5 clone 7-1 and then irradiated at varying doses. (Figure II-2) Radiation doses of 2, 4, and 6 Gy were used. Severe toxicity was observed with the virus infection alone. Due to this severe toxicity no further sensitization could be detected.

Figure II-2



Survival curves for control and iNOS infected DU-145 Cells

Analysis of cell growth was performed to determine the effect of virus infection at higher MOI and shorter time periods. Either DU-145 or PC3 cells were seeded into 35 mm tissue culture dishes at a concentration of 100,000 cells per dish and incubated at 37°C for 24 hours. The cells were infected with iNOS-AD5 clone 7-1 virus at MOI of 10 or 20. Phase-contrast photographs were taken at subsequent intervals of 24, 48, 72 or 96 hours. The results are shown in Figure II-3 for DU-145 and Figure II-4 for PC3 cells. The Du-145 cells had some rounding of cells after 1 day for both 10 and 20 MOI. The cell rounding continued through days 2 and 3. At 20 MOI the cells were becoming pyknotic at 3 days. After 4 days, cells infected with 10 MOI were also becoming pyknotic. DU-145 cells infected at 20 MOI were undergoing lysis at day 4.

PC3 cells infected with iNOS-AD5 clone 7-1 virus at MOI of 10 or 20 showed no noticeable toxicity one day after infection. On day two after infection, the PC3 cells were more rounded than the control cells. On day 3 after infection, The cell density in the dish was noticeably less in both MOI 10 and 20 dishes and the cells were rounded and disintigrating. On day 4 after infection the cell number per dish was much less in both the MOI 10 and 20 dishes compared to control cell and to the previous day at the same infection. The cells remaining in the dish were fragmented and non-viable by phase contrast.

Cell survival summary

Both DU-145 and PC cells were observed to be sensitive to being killed directly by infection with iNOS-AD5 at infection densities as low as 10 MOI. This infectivity level threshold for cytotoxicity correlates well with the level of infectivity that produces increased nitric oxide release. The current results are not conclusive about the radiation sensitizing effects of iNOS-AD5. Experiments must be completed in which lower MOI are used prior to radiation. These experiments are ongoing and will be

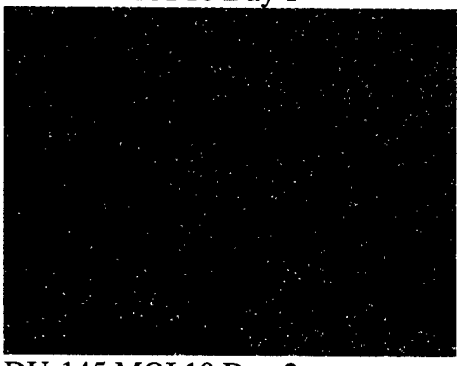
completed shortly. The critical range of MOI and time being investigated are those indicated by the nitric oxide release measurement results.

Figure 3

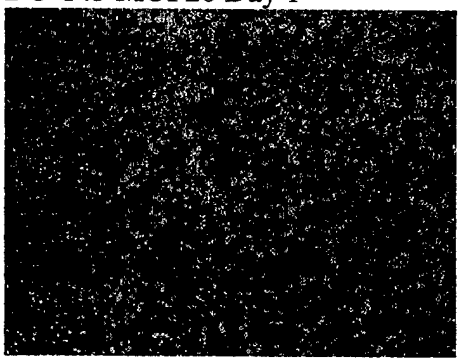
DU-145 cells were exposed to iNOS-AD5 clone 7.1 at MOI of 0, 10, or 20 for 1, 2, 3, or 4 day.

DU-145 MOI 0 Day 1

DU-145 MOI 10 Day 1

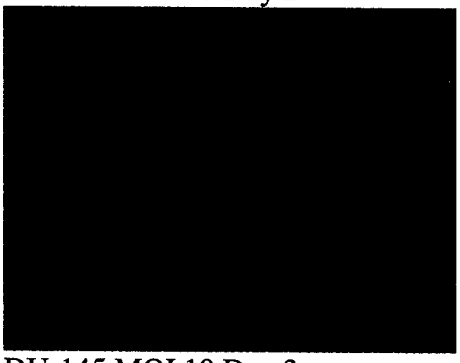


DU-145 MOI 20 Day 1

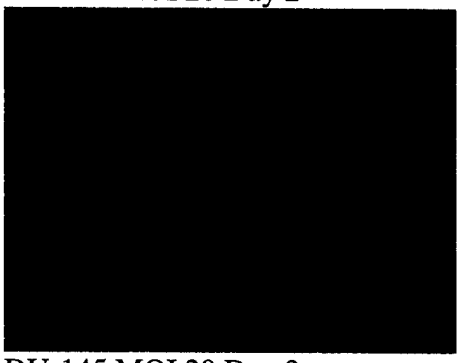


DU-145 MOI 0 Day 2

DU-145 MOI 10 Day 2

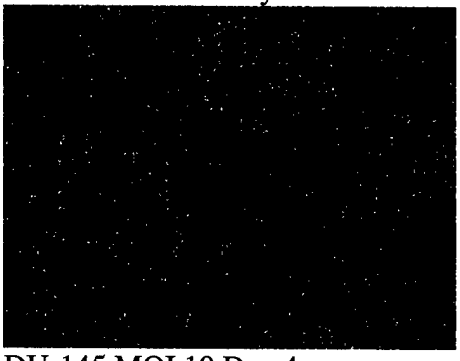


DU-145 MOI 20 Day 2

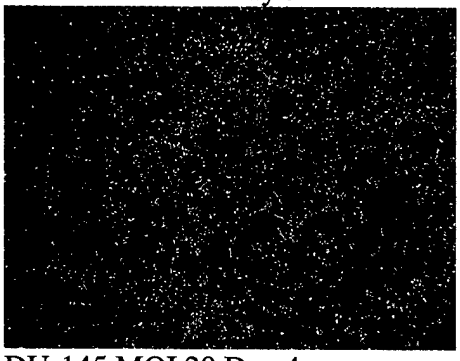


DU-145 MOI 0 Day 3

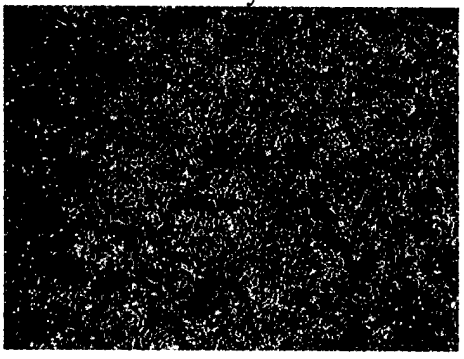
DU-145 MOI 10 Day 3



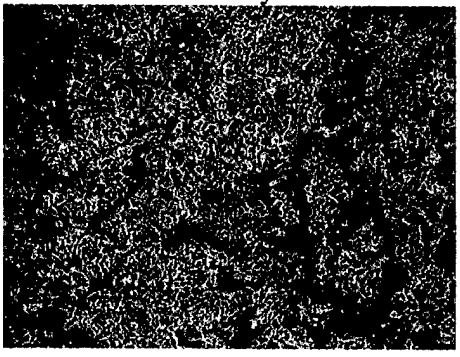
DU-145 MOI 20 Day 3



DU-145 MOI 0 Day 4



DU-145 MOI 10 Day 4



DU-145 MOI 20 Day 4

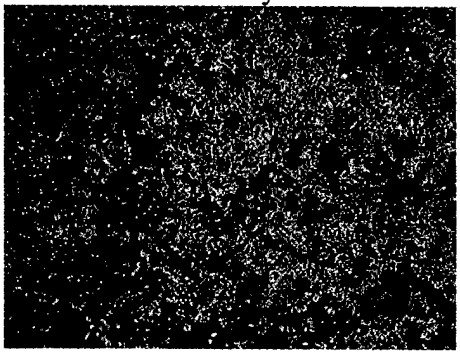
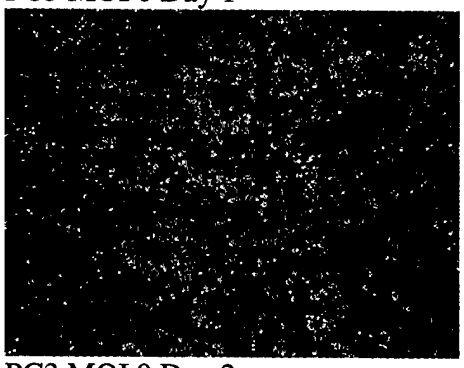


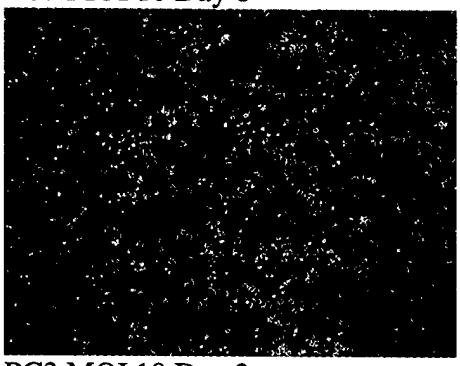
Figure 4

PC3 cells were exposed to iNOS-AD5 clone 7.1 at MOI of 0, 10, or 20 for 1, 2, 3, or 4 day.

PC3 MOI 0 Day 1



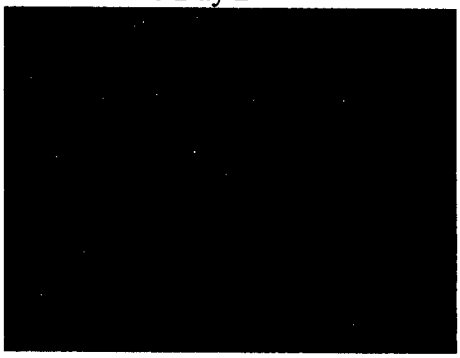
PC3 MOI 10 Day 1



PC3 MOI 20 Day 1

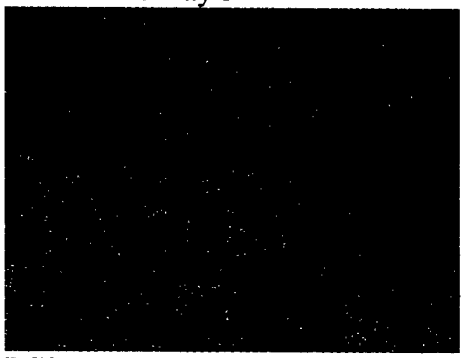
PC3 MOI 0 Day 2

PC3 MOI 10 Day 2

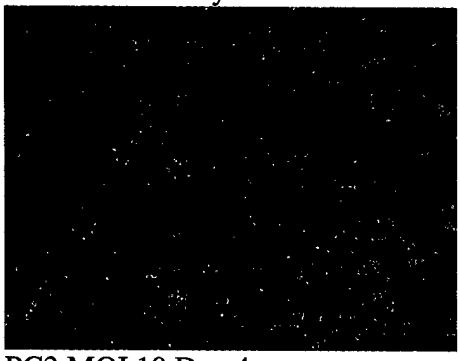


PC3 MOI 20 Day 2

PC3 MOI 0 Day 3

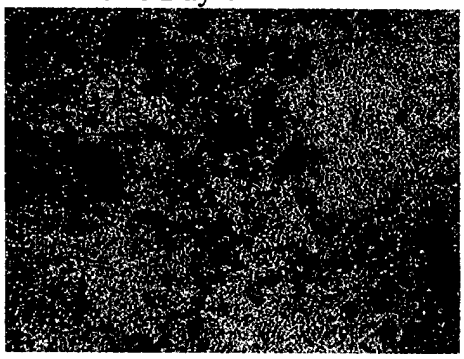


PC3 MOI 10 Day 3

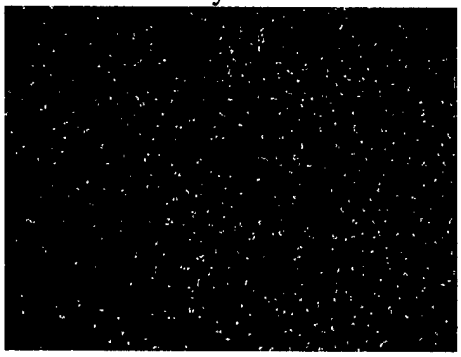


PC3 MOI 20 Day 3

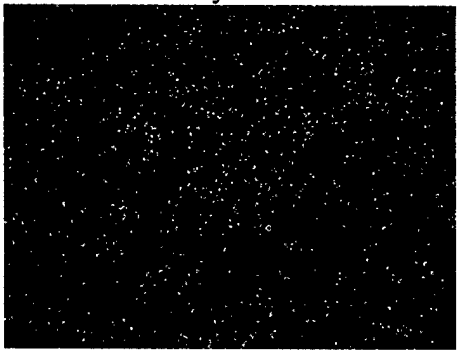
PC3 MOI 0 Day 4



PC3 MOI 10 Day 4



PC3 MOI 20 Day 4



Development of Mouse iNOS Adenovirus (Ad TG-N-miNOS)

An iNOS viral vector was developed from a mouse source to allow superior expression of iNOS and for future differentiation from endogenous expression of human iNOS. Clones of this virus have been tested for nitrite, nitrate and nitric oxide production.

Total RNA

Total RNA was isolated from mouse monocyte-macrophage RAW 264.7 cells (ATCC# TIB 71). They were stimulated to transcribe iNOS mRNA by adding medium containing 2ug/ml LPS (O26:B6) [Sigma] and 100units/ml rhIFN-gamma [Promega] to confluent cell cultures and incubating overnight. Total RNA was isolated from the cells using the STAT-60 method [Tel-Test]. This RNA was used as the target for a first strand cDNA synthesis. Figure II-5 presents a map of mouse iNOS cDNA showing important cut sites and primer sites we used for amplification and to identify length of final product.

First Strand cDNA

Using the SuperScript Preamplification System for First Strand cDNA Synthesis [Life Technologies] RNA/Primer mixture was prepared (10ul of total RNA 1ug/ul; 2ul of Oligo(dT) 0.5ug/ul; 12ul ddH_2O), divided into two 0.5ml PCR thin walled tubes and heated at 70°C for 10 min. PCR Reaction Mixture (9ul of 10X PCR buffer; 9ul of 25mM $MgCl_2$; 4.5ul of 10mM dNTP mix; 9ul of 0.1M DTT) was heated to 50°C, added to the RNA/Primer mix (14ul/tube) and incubated at 50°C for 5 min. To each tube, 2 ul (400 units) of SuperScript II RT was added, incubated at 50°C for 50 min., heated at 70°C for 15 min., and the RNA was digested with 2ul RNAse H (2 units/ul) at 37°C for 30 min.

Amplification of Target cDNA

The first strand cDNA iNOS template was amplified using Taq DNA polymerase [Promega]. The Primer mix was prepared (2ul of 10mM dNTP mix ; 2ul of 10uM forward (sense) primer; 2ul of 10uM reverse (antisense) primer; 2ul of first strand cDNA from mixture above; 32ul ddH_2O)

Sense primer named MINOSS2C [Genosys] ACCTTGGTGAAGGGACTGAG
Antisense primer named MINOSASC [Genosys] TGGAACTCTGGGCTGTCAGA

To this was added the PCR mix (10ul of 10X PCR buffer; 6ul of 25mM MgCl2; 1ul of Taq DNA polymerase; 43ul of ddH_2O) and the solution was amplified on a Thermal Cycler under the following conditions:

1 cycle	Hot start	94°C for 30s
35 cycles	Denaturation:	94°C for 30s
	Annealing:	55°C for 30s
	Extension:	68°C for 4 min
1 cycle	Cooling	4°C for up to 72 hrs

Product was run on a 1% agarose gel, excised and purified using the QIAEX gel extraction method [Qiagen]. The final product, GS2CASC-Taq (3.59 kb), was about 50 ng/ul.

Plasmid Construction

pTargeT Vector

The purified PCR product was ligated into the pTargeT Vector (5.67kb), transformed into JM109 competent cells and plated according to the pTargeT Mammalian Expression Vector System protocol [Promega]. White colonies were grown and plasmids purified using Wizard Plus Minipreps [Promega]. The plasmids were cut at the single Bgl II restriction site and screened for proper size (9.26 kb) on an agarose gel. Out of 24 clones only one, Clone 7, appeared to be the right size. To confirm proper orientation the plasmid, pTargeTmiNOSGS2CASC7, was cut with BamHI. Correct orientation would give fragments of 8502bp and 758bp. Reverse orientation would give fragments of 6373bp and 2887bp. When run on a gel the cut plasmid appeared to have fragments of about 9kb and 650bp. When the plasmid was subsequently transfected into 293 cells it produced nitrite readings on the Griess Assay of 8-10 times that of the control pSVBgal plasmid.

pZeroTG Vector

The pTargeTmiNOSGS2CASC7 plasmid was cut with Bgl II and Not I to produce two fragments, the desired fragment (4883bp) which included the CMV promoter from the pTargeT vector (the same as in pCINeo) attached to the front of the original miNOS insert, and another fragment 4377bp long. The fragments were separated on an agarose gel. The desired fragment was excised and extracted with the QIAEX gel extraction kit. The pZeroTG vector was cut with BamHI and Not I to produce a 3.6kb fragment, incubated with shrimp alkaline phosphatase (SAP), run on an agarose gel, excised, and extracted. The two fragments were then ligated with T4 DNA Ligase and plated on kanamycin LB agar dishes (pZeroTG has kanamycin resistance but no ampicillin resistance). Resulting colonies were grown in a tube of kanamycin LB broth and in a tube of ampicillian LB broth. Only colonies which grew in kanamycin but not in ampicillin were screened further. (Ampicillin resistance would mean the wrong (4377bp) fragment was ligated into the pZeroTG vector.) Clones now called pZTG/CMVmiNOS were cut with Bgl II to give a single fragment of 8463bp, and cut with EcoRI to give fragments of correct orientation 4148bp, 3072bp, 936bp, 307bp or reverse orientation 5295bp, 1925bp, 936bp, 307bp. All five clones tested were correct so Cl 4 was used.

Bacterial Recombination with pTGCMV (Wymann)

pTGCMV Cl2 was cut with Cla I, pZTG/CMVmiNOS Cl4 was cut with 4 different combinations of enzymes: Pac I + Bgl II (fragments 6155bp,2308bp); Pac I + Sph I (6868bp,1595bp); SgrAI + Bgl II (5965bp,2498bp); SgrAI + Sph II (6678bp,1785bp). BJ5183 competent bacteria were co-transformed with 100ng Cla I cut pTGCMV and 450ng cut pZTG/CMVmiNOS Cl4 (4 combinations) and plated on ampicillin LB agar plates (pTGCMV carries the ampicillin resistance) Three clones from each combination were grown in LB broth and purified for a total of 12 clones. Clones cut with Spe I were checked with PCR using primers at the start (MINOSS2C, MINOSAS2), the middle (MINOSSS4, MINOSAS4), and the end (MINOSSS5, MINOSASC) of the miNOS gene insert. Every clone except Cl4 was positive for all three locations although the start was much weaker on all of them.

The pCINeo CMV promoter from the pTargeT vector was preferred to the CMV promoter from the pTGCMV plasmid. It was determined that a plasmid which had a crossover before or near the beginning of the CMV promoter would keep the pCINeo CMV promoter and the plasmid would cut in 2 places with Spe I. Plasmids which kept the pTGCMV promoter would only cut once with Spe I. Clones 1, 2, 8, and 9 had two bands when cut with Spe I and run on an agarose gel. (So really only pZTG/CMVmiNOS Cl4 plasmids cut with Bgl II at the end for recombination were actually used). Those clones were transformed into DH5α cells, plated on ampicillin LB agar plates and colonies were grown in 2 ml Terrific broth the following day.

Cl 8 →clone 1 ; Cl 9 → clone 2 ; Cl 1 →clones 3, 4, 5 ; Cl 2 → clones 6, 7, 8, 9

Transfection for Virus Generation

Plasmids were purified using the Quantum Prep Plasmid Miniprep Kit [Biorad]. Clones 1-9 of pTG-N-miNOS (40kb) cut correctly with Pac I (fragments 38kb, 2kb) and the cut plasmids were precipitated in ETOH and resuspended in 0.1X sterile TE. 293 cells that were 80% confluent in a 5 cm culture dish were transfected using the Ca-Phosphate method with 1 ug of Pac I digested pTG-N-miNOS DNA and 10 ug of salmon sperm DNA as a carrier. Cells were transfected overnight in DMEM:F12 10% BCS with 2mM L-NAME and the next day the medium was removed and replaced with a 0.5% agarose overlay containing DMEM with 2% horse serum and 2mM L-NAME. One dish per clone was transfected. Viral plaques of Ad TG-N-miNOS were collected starting at 2 weeks. 293 cells in 6 well plates were infected from the plaques and virus was collected after 4-7 days by treating the concentrated 293 cells with multiple freeze – thaw cycles to lyse the cells and storing the supernatant in 10% glycerol.

Screening for function

Viral clones were screened for function on 293 cells plated at 70-80% confluence in 12 well plates. Cells were infected with 15ul of virus in 150ul of medium at 37°C for 1 hour , then 1 ml of growth medium was added and medium was collected after 4 days. A Griess Assay was performed on the cell medium after treating the medium with 1.5% zinc sulfate to precipitate protein and with cadmium to convert nitrate to nitrite (Figure II-6). Clones were also tested on PC3 and DU-145 cells for nitrite production with the Griess Assay (Figure II-7). Selected viral clones are being grown and measured on plaque assays to determine the number of plaque forming units (PFU).

Figure II-5 miNOS AD5 virus construction

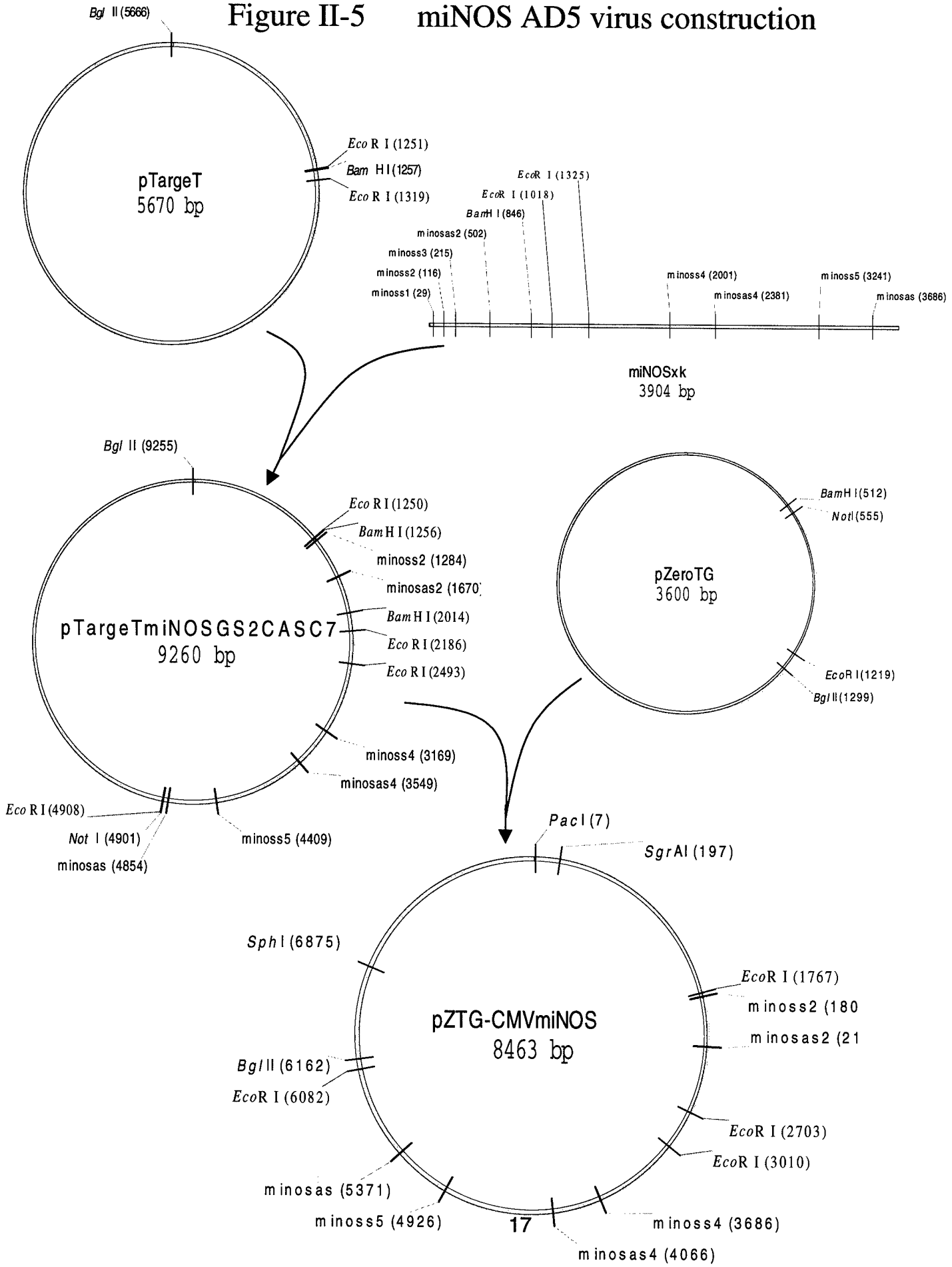


Figure II-6

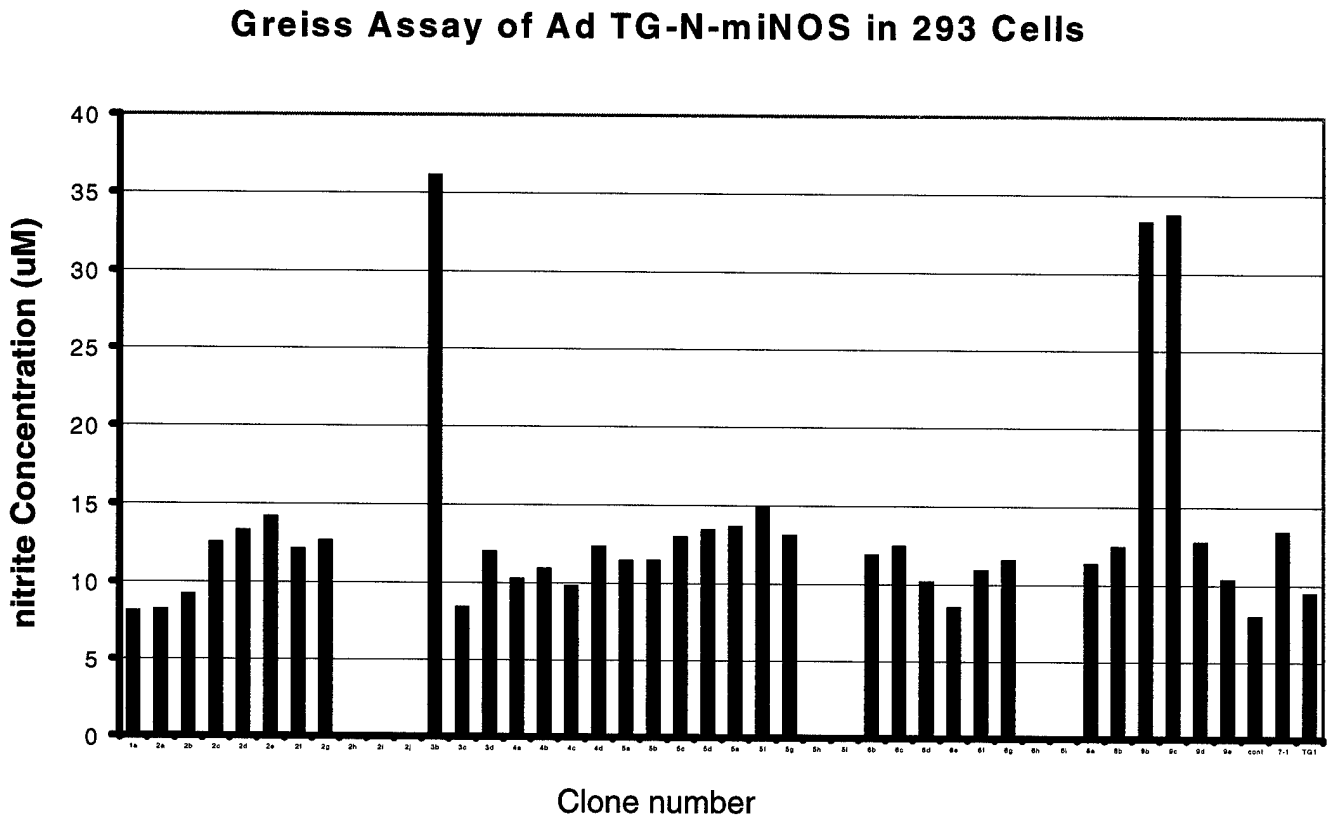
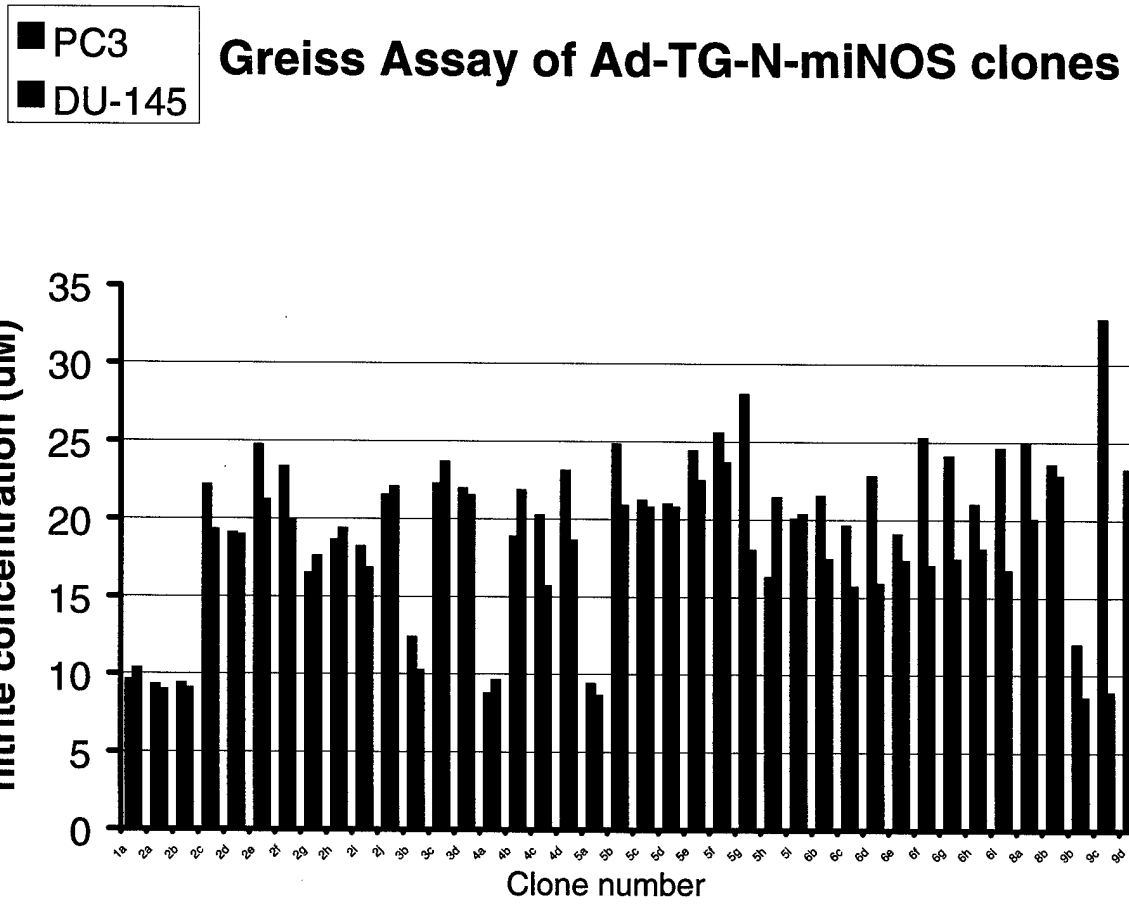
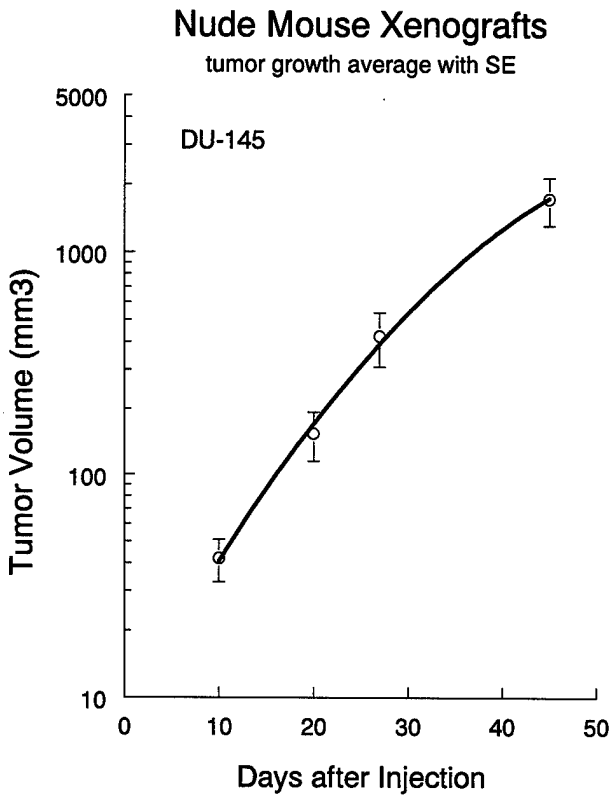


Figure II-7



Task III ....Determine the distribution of iNOS expression and O, $O^{\bullet -}_2$, H_2O_2, and $ONOO^-$ production after Ad5-CMV-iNOS infection of DU-145 and PC-3 cells growing as tumors in nude mice. The porphyrin microelectrodes, immunohistochemistry, Griess assay, luminescent and HPLC will be used for analysis.

Figure III-1



Initial experiments were performed to establish the growth rates of DU-145 cells growing as xenograft tumors in nude mice under our current conditions, and confirm the tumor cell implant experimental conditions. The growth rate for three tumors averaged is presented in Figure III-1. The small group (12) of mice which were to be the first batch of mice used for a viral infection experiment became sick prior to being entered into the experiment. The problem with the mice was either derived from the vender of the mice or a mechanical failure at the Beaumont Research Services facility. The conditions have been thoroughly reviewed and I have received assurance that the problems have been alleviated. Because this problem has never occurred previously, I am confident that no further delay of the xenograft tumor experiments will occur. We have in-fact recently initiated tumors in mice and will be performing the first tumor infections with iNOS adenovirus within two weeks.

The Radiation Oncology Department is currently purchasing the OxyLite Photometric fiber optical system that is capable of continuously measuring oxygen concentration in tumor or normal tissues for several hours. The OxyLite system will be available for use in this project within 2 months. It will enhance our ability to monitor changes in tumor hypoxia during the course of expression of iNOS adenovirus in xenograft tumors. The OxyLite has been successfully applied to tumor oxygen tension measurement in murine and human tumors. There two advantages of this instrument that will greatly enhance our analysis of iNOS Adenovirus modulation of hypoxia and thus tumor radiation sensitization. The first is that the probes are precalibrated and stable over time thus allowing long term monitoring of oxygen tension during virus expression in the tumor. The second is that the probes have optimal sensitivity in the oxygen tension range of 0 to 10 mm Hg which is the range most critical for tumor cell resistance due to hypoxia. The utilization of this system will not change the original statement of work, but will enhance the efficiency and precision of the experiments performed to complete the originally proposed work.

(7) Key Research Accomplishments

- Optimal nitric oxide production by DU-145 prostate cancer cells infected with iNOS-AD5 clone 7-1 occurred at MOI 15 and in the time range between 24 and 36 hours.
- The CMV promoter on iNOS-AD5 clones 7-1, 7-3, 7-4, 7-6, and 9-7 effectively promotes production of nitric oxide but is not further induced by heat shock.
- Infection of prostate cancer cells with iNOS-AD5 clone 7.1 produces direct cytotoxicity as measured by colony formation at MOI greater than 5.
- Observable cytotoxicity after infection with iNOS-AD5 clone 7.1 at MOI 10 or 20 is initiated as early as day one after infection in DU-145 prostate cancer cells and is severe with partial lysis by day four after infection.
- Cytotoxicity by infection of PC3 prostate cancer cells with iNOS-AD5 clone 7.1 was not observed until day 3 but the toxicity proceeded to severe by day four after infection with almost complete lysis at MOI 10 or 20.
- A new set of viral clones expressing mouse iNOS produces at least as much nitric oxide as iNOS-AD5 clone 7.1.
- Large clonal variation must be considered when choosing viral clones for use in expression and cytotoxicity experiments.

(8) REPORTABLE OUTCOMES:

- development of cell lines, tissue or serum repositories;

  Several adenovirus vector clones that express the mouse iNOS cDNA sequence and produce mouse iNOS protein were developed.

- employment or research opportunities applied for and/or received on experiences/training supported by this award

  A Radiation Oncology medical resident (John Register, M.D.) received research training required for completion of his residency in association with this project. (no financial support was provided to Dr. Register)

  A high school student (Priya Gopwani) received research training for 3 months prior to entering the University of Michigan pre-med program.

(9) CONCLUSIONS:

The nitric oxide measurement experiments have demonstrated that the iNOS-AD5 clone 7.1 vector produces a significant amount of nitric oxide in DU-145 cells and that the time course and MOI must be controlled to result in optimal nitric oxide release. Nitric oxide release increased with increasing MOI up to 15 where after a decrease was observed. The nitric oxide release rate also depended upon time of incubation after infection. The maximum release rate was observed at 24 to 36 hours after infection. Combined with the observed cellular toxicity results we conclude that the decrease in nitric oxide release rate above MOI 15 and time of 36 hours is due to loss of macromolecular synthesis and general cellular metabolism as the cells die. The results also indicate that the new mouse iNOS vector miNOS-AD5 produces NO at least as well as the 7-1 iNOS-AD5 clone. The emphasis in future in vivo experiments will be to focus on events occurring in the 24 to 48 hour time period after infection.

The CMV promoter on iNOS-AD5 clones 7-1, 7-3, 7-4, 7-6, and 9-7 effectively promoted production of nitric oxide but was not further induced by heat shock. The accumulation of NO2 + NO3 in all iNOS-AD5 infected cells was significantly above the control level but no differences between heated and non-heated were observed in any of the five clones. The CMV promoter will be the promoter of choice in future iNOS adenovirus vector experiments but we will continue to analyze the role of clonal and CMV promoter variation.

Both DU-145 and PC3 prostate cancer cells were observed to be sensitive to being killed directly by infection with iNOS-AD5 at infection densities as low as 10 MOI. This infectivity level threshold for cytotoxicity correlates well with the level of infectivity that produces increased nitric oxide release. The time course over which iNOS-AD5 produces cytotoxicity in prostate cancer cells was to be in the range of 1 to 4 days. This rate is more rapid than that observed with radiation and some chemotherapy agents. The relatively rapid loss of cells will be important to managing the consequences of tumor therapy. The current experimental results are not conclusive about the radiation sensitizing effects of iNOS-AD5. Experiments must be completed in which lower MOI are used prior to radiation. These experiments are ongoing and will be completed shortly. The critical range of MOI and time being investigated are those indicated by the nitric oxide release measurement results.

Initial experiments were performed to establish the growth rates of DU-145 cells growing as xenograft tumors in nude mice under our current conditions, and confirm the tumor cell implant experimental conditions. Subsequently a problem with the health of a batch of mice caused a temporary delay in *in vivo* progress. The situation has been resolved and I am confident that no further delay of the xenograft tumor experiments will occur. We have in-fact recently initiated tumors in mice and will be performing the first tumor infections with iNOS adenovirus within two weeks.

(10) REFERENCES

1. Graham, F. L. and Prevec, L. Methods for construction of adenovirus vectors, Mol Biotechnol. 3: 207-220, 1995.
2. Park, C. S., Park, R., and Krishna, G. Constitutive expression and structural diversity of inducible iso-form of nitric oxide synthase in human tissues, Life Sci. 59: 219-25, 1996.
3. Malinski, T. and Taha, Z. Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor [see comments], Nature. 358: 676-8, 1992.
4. Nathan, C. and Xie, Q. W. Nitric oxide synthases: roles, tolls, and controls, Cell. 78: 915-8, 1994.
5. Schmidt, H. H. H. W. W., Ulrich NO at Work, Cell. 78: 919-925, 1994.
6. Nathan, C. Nitric oxide as a secretory product of mammalian cells, Faseb J. 6: 3051-64, 1992.
7. Knowles, R. G. and Moncada, S. Nitric oxide synthases in mammals, Biochem J. 298: 249-58, 1994.
8. Nathan, C. and Xie, Q. W. Regulation of biosynthesis of nitric oxide, J Biol Chem. 269: 13725-8, 1994.
9. Mitchell, J. B., Cook, J. A., Krishna, M. C., DeGraff, W., Gamson, J., Fisher, J., Christodoulou, D., and Wink, D. A. Radiation sensitisation by nitric oxide releasing agents, Br J Cancer. 74: S181-S184, 1996.
10. Mitchell, J. B., Wink, D. A., DeGraff, W., Gamson, J., Keefer, L. K., and Krishna, M. C. Hypoxic mammalian cell radiosensitization by nitric oxide, Cancer Res. 53: 5845-5848, 1993.
11. Howard-Flanders, P. Effect of Nitric Oxide on the Radiosensitivity of Bacteria, Nature. 180: 1191-1192, 1957.
12. Dewey, D. L. Effect of Oxygen and Nitric Oxide on the Radiosensitivity of Human Cells in Tissue Culture, Nature. 186: 780-782, 1960.
13. Moncada, S., Palmer, R. M., and Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharma-cology, Pharmacol Rev. 43: 109-42, 1991.
14. Griffin, R. J., Makepeace, C. M., Hur, W. J., and Song, C. W. Radiosensitization of hypoxic tumor cells in vitro by nitric oxide, Int J Radiat Oncol Biol Phys. 36: 377-383, 1996.
15. Verma, I. M. and Somia, N. Gene therapy -- promises, problems and prospects [news], Nature. 389: 239-42, 1997.
16. Graham, F. L. and Prevec, L. Adenovirus-based expression vectors and recombinant vaccines, Biotech-nology. 20: 363-390, 1992.
17. Graham, F. L. and Prevec, L. Manipulation of Adenovirus Vectors. In: E. J. Murray (ed.) Methods in Molecular Biology, pp. 109 - 128, 1991.
18. Graham, F. L. Adenoviruses as expression vectors and recombinant vaccines, Trends Biotechnol. 8: 85-87, 1990.
19. Wishnik, J. S., Glogowski, J. S., and Tannenbaum, S. R. Quantitation of Nitrate, Nitrite, and Nitro-sating Agents. Methods in Enzymology, Vol. 268, pp. 130-145, 1996.
20. Vodovotz, Y. Modified microassay for serum nitrite and nitrate, Biotechniques. 20: 390-2, 394, 1996.
21. Xie, K., Huang, S., Dong, Z., Juang, S. H., Wang, Y., and Fidler, I. J. Destruction of bystander cells by tumor cells transfected with inducible nitric oxide (NO) synthase gene [see comments], J Natl Cancer Inst. 89: 421-7, 1997.
22. Xia, Y. and Zweier, J. L. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages, Proc Natl Acad Sci U S A. 94: 6954-8, 1997.
23. Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. Nitric oxide synthase gener-ates superoxide and nitric oxide in arginine- depleted cells leading to peroxynitrite-mediated cellular injury, Proc Natl Acad Sci U S A. 93: 6770-4, 1996.
24. Leith, J. T., Quaranto, L., Padfield, G., Michelson, S., and Hercbergs, A. Radiobiological studies of PC-3 and DU-145 human prostate cancer cells: x-ray sensitivity in vitro and hypoxic fractions of xeno-grafted tumors in vivo, Int J Radiat Oncol Biol Phys. 25: 283-7, 1993.
25. Vreeke, M. S., Maidan, R., and Heller, A. Hydrogen peroxide and B-nicotinamide adenine dinucliotide sensing amperometric electrodes based onelectrical connection of horseradish peroxidase redox centers to electrodes through a three dimensional electron relaying polymer network, Anal. Chem. 64: 3084-3090, 1992.
26. Malinski, T. and Czuchajowski, L. Nitric oxide measurements by electrochemical methods. In: M. Feelisch and J. S. Stamler (eds.), Methods of nitric oxide research, pp. 319-389. Chichester, U. K.: John Wiley, 1996.
27. Mesaros, S., Grunfeld, S., Mesarosova, A., Bustin, D., and Malinski, T. Determination of nitric oside saturated (stock) solution by chronoamperometry on a porphyrine microelectrode, Anal. Chim. Acta. 339: 265-270, 1997.