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RESEARCH ARTICLE

Functional expression of ATM gene carried by HSV amplicon vector in vitro and in vivo

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Ataxia-telangiectasia (AT) is a human autosomal recessive disease with a pleiotropic phenotype characterized by cerebellar degeneration, immunodeficiency, premature aging, cancer predisposition, and radiation sensitivity. The gene mutated in AT, ATM (for AT-mutated), had been cloned and found to have ionizing radiation and oxidative stress-inducible kinase activity. No treatment can stop the progression of the disease. In this study, the complete open-reading frame of ATM cDNA was cloned into a Herpes simplex virus type-1 (HSV-1) amplicon vector (pTO-ATM), and the transduction of cultured AT cells was demonstrated by immunohistochemistry and Western blot analysis. Functional gene expression was evaluated by cell colony-forming assays following exposure to oxidative stress. The survival of AT cells with ATM gene transduction was about 100% higher compared to nontransduced cells after t-butyl hydroperoxide treatments. Next, the normal ATM gene expression in different regions of the rat brain was studied. Immunohistochemistry staining demonstrated weak endogenous ATM protein expression in neurons of the caudate-putamen, with significantly higher levels of expression detected in neurons in other brain regions. Exogenous ATM gene expression from pTO-ATM after viral transduction in the caudate-putamen of the adult rat was examined. At 3 days after injection of the pTO-ATM viral vector, abundant positive ATM staining of the neurons was found at the injection sites, in comparison to the controls. These data demonstrate that the relatively large ATM cDNA can be transduced and expressed in vitro and in vivo from an HSV amplicon viral vector. These data provide initial evidence that the replacement of the ATM gene into the cells of AT patients might be possible some day.
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Introduction

Ataxia-telangiectasia (AT) is a human autosomal recessive disorder characterized by a wide variety of clinical manifestations, including oculocutaneous telangiectases, immune deficiency, growth retardation, increased radiosensitivity, premature aging, and increased cancer rates. AT patients exhibit an approximately 1000-fold elevated rate of blood malignancies compared to the general population. One of the clinical hallmarks of the disease is progressive cerebellar degeneration, leading to truncal and limb ataxia and abnormal eye movement. Usually, AT patients are unable to walk and are confined to wheelchairs by their early teens.[1–4]

The AT locus has been mapped to human chromosome 11q22–23 8 and the gene responsible for AT, called ATM (AT-*mutated*), has been identified and cloned.[5,6] The isolation of the ATM gene has made it possible to study the gene product, its functions, and possible ways to treat the disease. Sequence analyses have revealed that the ATM gene product is a polypeptide around 350 kDa that localizes to the nucleus and within vesicular structures in the cytoplasm.[5–8] The exact role of the ATM protein is not yet known. However, based on the pleiotropic phenotype of AT and its close relationship to a family of phosphatidylinositol (PI) 3-kinases, it is linked to signal transduction, cell cycle control, DNA repair-related mechanisms, and resistance to oxidative stress.[9–17]

No effective methods have been found to stop the progressive and unrelenting course of AT or improve the impaired cellular function. Therefore, gene therapy is being considered as a potential therapeutic method. With this in mind, no prior reports have shown effective ATM gene transducton in experimental animals. One reason for this is that the 9.2 kb ATM gene cDNA is beyond the packaging capacity of most currently available viral vectors including retroviral and classic adenoviral vectors. The other reason is that the ATM gene is unstable and it is difficult to get high levels of gene expression in targeted cells both *in vitro* and *in vivo*.

Herpes simples virus type-1 (HSV-1) vectors have demonstrated efficient gene transfer *in vitro* and *in vivo*.[18–23] Several ongoing human clinical trials are based on HSV vectors.[24–26] One of the two broad categories of HSV-based vectors, the amplicon, is a plasmid-type vector, which carries an HSV-1 lytic replication origin (ori_s) and HSV-1 packaging signal sequence. The amplicon can be amplified and packaged into infectious HSV-1 virions in the presence of the helper virus.[27–29] This plasmid system permits easier cloning, and carries

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genomic information between procaryotic and eucaryotic cells as a shuttle vector.[30] The amplicon system retains the merits of HSV-1 vectors including high efficiency of gene transfer *in vitro* and *in vivo*, a wide range of hosts, and neurotropism.[31–38] In this study, a HSV-1 pTO amplicon vector, pTO-ATM, was used to carry the 9.2 kb ATM cDNA by viral vector transduction into ATM-negative cells. The pTO-ATM viral vector was then used to transduce neurons by directly injecting the viral stock into rat brains. ATM gene expression and its duration were shown within the rat caudate-putamen by immunohistochemistry.

Results

pTO-ATM amplicon vector

We constructed an HSV amplicon vector, pTO-ATM, which carries the 9168 bp ATM open-reading frame (ORF), driven by a human cytomegalovirus (CMV) immediate-early promoter (Figure 1). The vector is 13 kb in size and was demonstrated to stably carry an intact ATM cDNA. The vector also contains HSV-1 the package/cleavage signal and an ori_s replication origin for the replication packaging of the plasmid, with the presence of helper virus (5dl1.2) in 2–2 helper cells. The ratio of titers between amplicons and viruses ranged from 1:5 to 1:20.

ATM gene expression in cultured cells

To confirm the expression of ATM, AT5BIVA cells (AT gene mutated cells) were infected with pTO-ATM viral vector at various multiplicities of infection (MOIs). After 24 h, ATM gene expression in the cells was monitored by immunohistochemistry. We chose this time point as time course analysis indicated that ATM protein expression was high at this point (data not shown). ATM-expressing cells were stained strongly in the nucleus and moderately in the cytoplasm (Figure 2a). The number of positively staining cells increased, based upon the MOI used for transduction. At 0.1 MOI, positively stained cells are clearly distinguished from the neighboring cells (Figure 2a). Only background staining was observed in AT5BIVA cells that were not transduced (Figure 2b). Some positively stained cells were found in VA13 cells, which contain a normal ATM gene and served as a positive control (Figure 2c). The transgene expression with pTO-ATM vectors was also demonstrated by Western analysis (Figure 3). The recombinant pATM

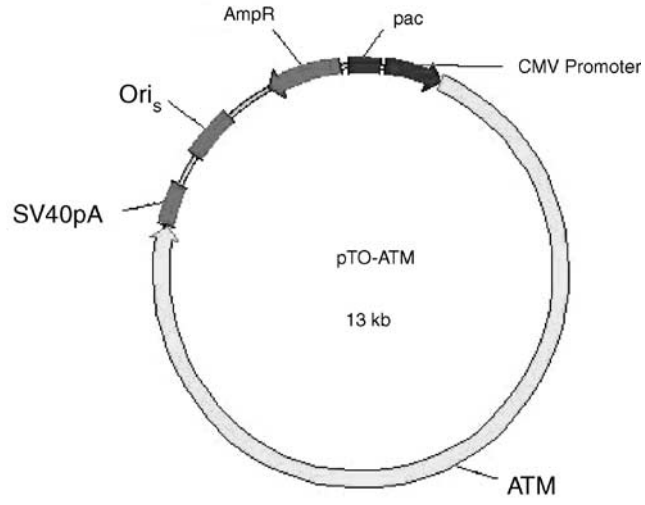

Figure 1 *Construction of the pTO-ATM plasmid. AmpR: ampicillin resistance gene; ATM: AT-mutated gene; CMV promoter: CMV immediate-early promoter; ori$_s$: HSV-1 replication origin; pac: HSV-1 packaging/cleavage signal; SV40 poly A: Simian virus 40 polyadenylation signal.*

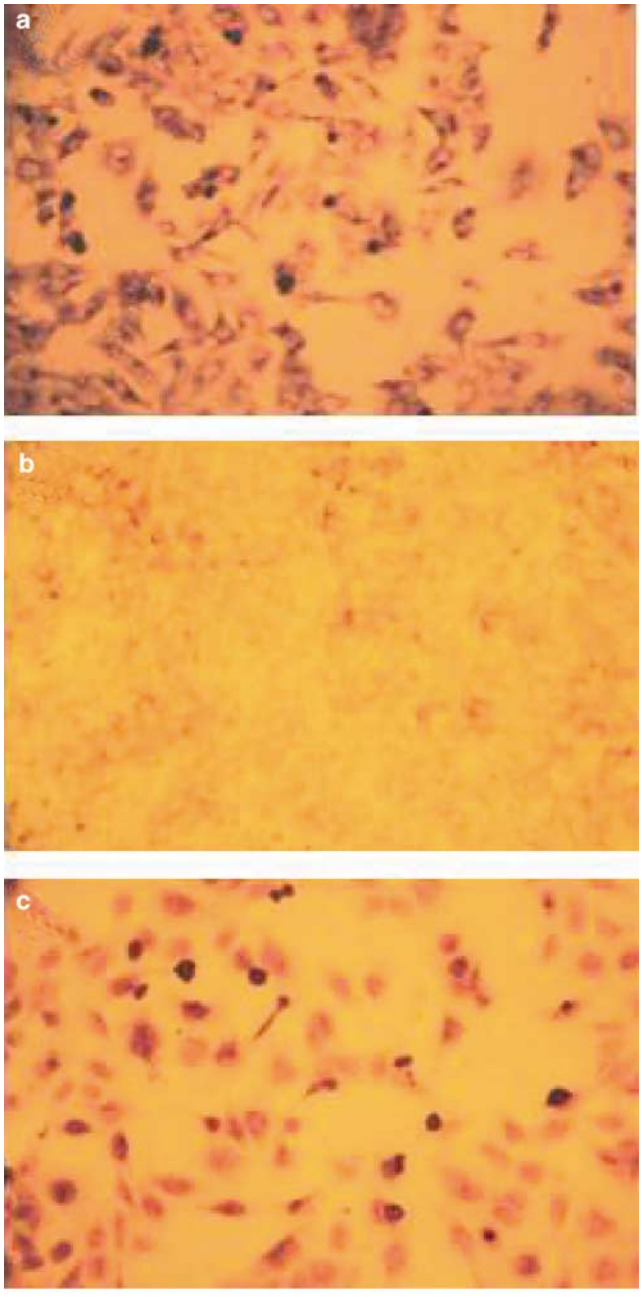

Figure 2 *Expression of the ATM gene in cultured cells after transduction with the pTO-ATM amplicon vector. Immunohistochemistry was performed by using goat primary antibody (1:100) and rabbit anti-goat secondary antibody conjugated with alkaline phosphatase (1:100). (a) AT5BIVA cells, 24 h after transduction (0.1 MOI). (b) AT5BIVA cells without transduction. (c) VA13 cells, ATM gene-positive cells. Magnification: × 200.*

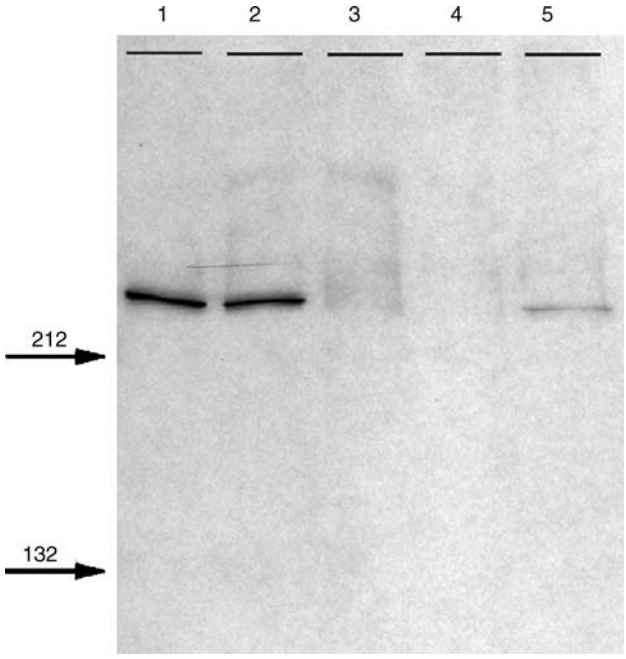

Figure 3 *Immunoprecipitation (IP)-Western analysis of AT cells 24 h after transduction by the pTO-ATM vector. Lane 1, VA13 (positive control); lane 2, YZ5 (positive control); lane 3, AT22 (negative control); lane 4, AT22 transduced with pTO-GFP (negative control for viral vector) at an MOI of 0.01; lane 5, AT22 transduced with pTO-ATM vector at an MOI of 0.01. Lanes 1 and 2 were IP against 0.32 mg and lanes 3–5 were IP against 1.28 mg of total cellular protein (1:4 ratio). Molecular marker weight: kDa.*

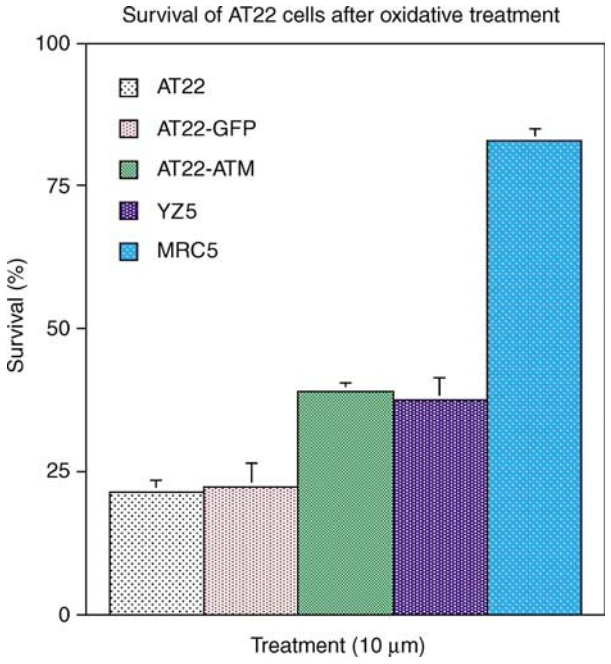

Figure 4 *Functional correction of AT cells after ATM gene transduction. Expression of recombinant pATM in the AT22 tumor cell line results in increased resistance to the toxic effects of t-butyl hydroperoxide in the colony-forming-efficiency assay. AT22 cells were either untreated or transfected with the pTO-GFP or pTO-ATM amplicon vectors at an MOI of 0.1 and cultured for 18 h, as described in the Materials and methods. The cells were treated with 10 μM t-butyl hydroperoxide for 15 min, the plates washed 2× with HBSS, cultured for 14 days, and stained. Data indicate survival as a percentage of untreated cells.*

gene expression in the AT cell line (AT22, lane 5) was increased following pTO-ATM gene transduction compared to untransduced cells (lane 3). Cells transduced with pTO-green fluorescent protein (GFP) (lane 4) exhibited essentially no ATM gene expression.

Functional correction of AT cells after ATM gene transduction

In order to test whether or not the ATM gene could function inside a virally transduced AT cell line, colony-forming-efficiency experiments were conducted. As shown in Figure 4, viral transduction of the ATM-deficient AT22 cell line with the pTO-ATM viral vector at an MOI of 0.1 resulted in an approximately 100% increase in cellular resistance to a 15 min *t*-butyl hydroperoxide exposure compared to nontransduced AT22 cells. The increased cellular survival seen in the pTO-ATM viral vector-transduced AT22 cells was specific for ATM gene activity, as transduction of the AT22 cell line with the control pTO-GFP viral vector failed to increase cellular survival over that of nontransduced cells following *t*-butyl hydroperoxide exposure. The degree of cellular resistance observed was far less than that of the ATM-normal MRC5 cell strain, and approximately equal to that seen in the YZ5 cell line. This latter cell line is an AT cell line that expresses a recombinant ATM gene product. The standard deviations (error bars, Figure 4) were evaluated within each experiment as a per cent of untreated, or untreated pTO-GFP- or pTO-ATM-transduced cells, and averaged between different experiments.

Distribution of ATM gene product in the rat forebrain and the cerebellum

Immunostaining experiments revealed that endogenous ATM protein is largely located in neuronal nuclei. However, the intensity of ATM staining and the shape of the stained nuclei were very diverse in different areas of the forebrain and the cerebellum. In the cerebral cortex, intense ATM-stained neurons were located in the piriform cortex (Figure 5a), the cortex–amygdala transition zone, and the islands of Calleja. The frontal and parietal cortexes had high levels of ATM-stained neurons, with strong staining in the fifth layer of the large pyramidal neurons (Figure 5b). In the basal ganglia, moderately stained neurons were observed in the nucleus accumbens, and weaker ATM staining was found in the neurons of the caudate-putamen (Figure 5c), globus pallidus, and claustrum. In addition, moderate to strongly stained neurons were also present in the medial preoptic area, the lateral preoptic area, and the amygdaloid nuclear group. In the cerebellar cortex, the Purkinje cells and the cells in the monolayer were strongly stained, whereas the cells of the granular layer were virtually devoid of labeling (Figure 5d). In addition, the cerebellar nuclei were also stained moderately (Figure 5e). After neutralizing the primary antibody with a working concentration by ATM peptide or substituting the primary antibody with phosphate-buffered saline (PBS), no positive ATM immunoreactive cells were found in the brain sections (Figure 5f).

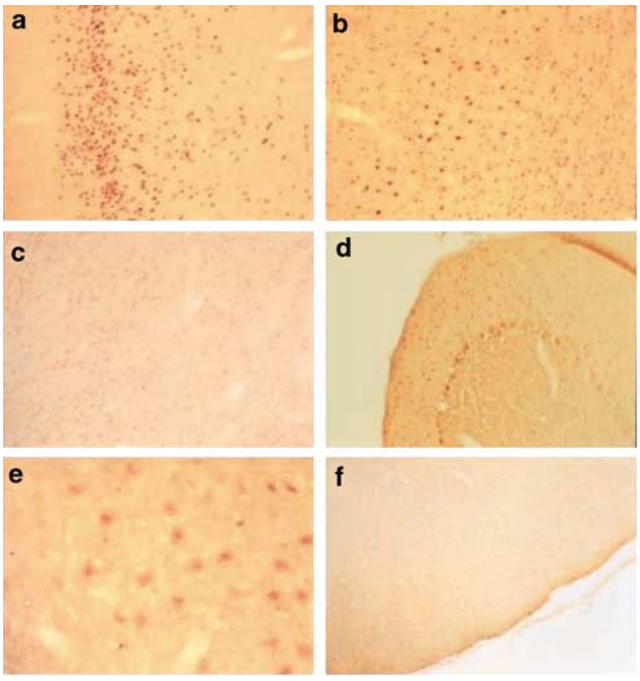

Figure 5 *Distribution of ATM product immunoreactivity in the rat forebrain and cerebellum. On 30 μm thick, free-floating brain sections, immunohistochemistry was performed by using goat primary antibody (1:300), biotinylated mouse monoclonal anti-goat secondary antibody (1:200) and avidin-biotinylated horseradish peroxidase (1:200). (a) Piriform cerebral cortex, ×200. (b) Frontal cerebral cortex, ×200. (c) Caudate-putamen, ×200. (d) Cerebellar cortex, ×200. (e) Cerebellar nucleus, ×400. (f) Cerebral cortex section after incubation with the primary antibody neutralized by ATM peptide, ×100.*

In vivo expression of ATM gene delivered into the caudate-putamen of the rat

To identify the expression of ATM gene transferred by our HSV pTO-ATM amplicon vector in animal brains, we stereotaxically injected 15 adult rats with 4 μl of pTO-ATM vector stock solution (amplicon titer: 8.7×10^7 infection units/ml) into the caudate-putamen of each rat. At different time points after the injection, five animals were anesthetized and anti-ATM protein immunohistochemistry was used to evaluate ATM expression in the rat brain. At 3 days after injection, abundant ATM staining was found in the injection site of the caudate-putamen, including the area surrounding the needle track (Figure 6a). We found no positive staining in the contralateral caudate-putamen. After substituting the primary antibody with PBS and incubating the neighboring sections of positively stained sections, no ATM staining was found in these sections after 3 days of injection (Figure 6b). At 7 days after injection, there were still some stained cells found in the injection site, although the number had decreased significantly (Figure 6c). At 13 days after injection, no stained cells were found in the caudate-putamen (Figure 6d).

Discussion

The ATM gene is one of the key components involved in cell cycle control and cellular defense systems, such as DNA repair. Dysfunction of the ATM gene causes a

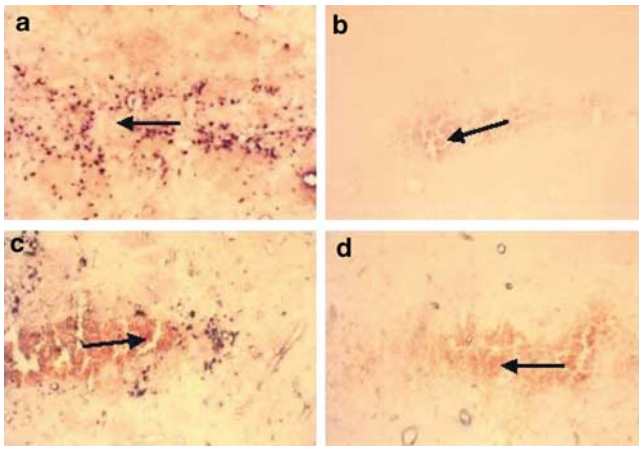

Figure 6 *Expression of the ATM gene in the caudate-putamen of the rat brain. After injection with 4 μl of pTO-ATM amplicon vector (total ATM viral particles of 3.5×10^5), the brains were postfixed and cryoprotected. On 10 μm thick brain sections attached on polylysine-coated slides, immunohistochemistry was performed by using goat primary antibody (1:100) and rabbit anti-goat secondary antibody conjugated with alkaline phosphatase (1:100). The arrows show the needle track. (a) Day 3 after injection with pTO-ATM vector. (b) Day 3 after injection with PBS control. (c) Day 7 after injection with pTO-ATM vector. (d) Day 13 after injection with pTO-ATM vector. Magnification: ×200.*

multisystem disorder in individuals with AT. Since the ATM gene was identified and cloned,[5,39,40] many attempts have been made to deliver the gene into cultured cells and *in vivo* to further study the functions of the ATM gene. The complete ORF of the ATM gene is 9.168 kb,[5] which is large and extremely unstable in many expression vectors. Dr Ziv *et al*[41] employed a bacterial plasmid pFastBac1 that was designed for the cloning of unstable sequences, and successively cloned a 4.6 and 4.95 kb cDNA of the ATM ORF gene into this plasmid. This group subsequently subcloned the inserts into a eucaryotic expression vector which contains the origin of replication of the Epstein–Barr virus, and expressed the ATM gene in SV40 virus-transformed AT cells and restored AT cells to a normal sensitivity to ionizing radiation and the radiomimetic drug neocarzinostatin.[41] Scott *et al*[42] cloned the complete full-length ATM cDNA into baculovirus vectors and expressed the ATM cDNA in insect cells. However, the efficiency of transfection of the DNA was very limited, especially in primary cultured cells and *in vivo*. *In vitro*, in order to conduct function analysis, a certain amount of cells being evaluated must be transfected. Ideally, artificially transfected normal and nontransfected cells would have uniform transgene expression level. Past researchers have used immortalized cell lines, but many of the biological functions are altered in such cell lines. In this regard, viral vector-mediated gene transfer has advantages over transfection methods, since up to 95–99% of a cell population can be efficiently transduced.[33,34] Viral vectors are even more efficient for gene transfer *in vivo*.[18,19,43–45] The full-length ATM cDNA is beyond the packaging capacity of most widely used viral vectors; so we employed an HSV-1-based amplicon vector that, in theory, can package 140 kb of DNA payload.[33,34] The ATM gene, therefore, was cloned into pTO HSV amplicon vector and the insert was confirmed to be intact by Southern blot analysis (data not shown). The

transgene expression was then demonstrated. In cultured AT cells, ATM expression was evaluated after viral vector transduction by immunohistochemistry and Western blot (Figures 2 and 3). At 0.1 MOI of transduction, about 10% of cultured AT cells stained positive for AT protein (Figure 2a). VA13 (SV40 virus transformed) normal human fetal lung fibroblast cells served as a positive control and demonstrated ATM gene expression (Figure 2c). However, the numbers of expressing VA13 cells were less than that observed in populations of pTO-ATM-transduced AT cells. This likely reflects that the pTO-ATM transduction produced the ATM gene expression regardless of cell cycle, and that multiple copies of the transgene were expressed. The positive staining of endogenous ATM protein in VA13 cells and the negative staining in AT cells that were not transduced verified the specificity of the immunostaining. ATM staining was located predominantly in the nucleus of the transduced AT cell, with lower levels in the cytoplasm as reported previously.[41] In Western blot analysis, ATM gene expression was found in ATM-negative cells after pATM gene transduction (Figure 3, lane 5). Although the expression level was not as high as the controls (Figure 3, lanes 1 and 2), due partially to the low MOI used for transduction, it certainly demonstrated that the pTO amplicon vector can carry, package, and express the ATM gene in cultured AT cells. The ATM protein could be detected 24 and 48 h after gene transduction.

To test whether or not the virally transduced ATM gene was capable of functioning inside the cell and correcting the functional defects of AT, the AT22 cell line was transduced with pTO-ATM at an MOI of 0.1. We were able to demonstrate that viral transduction of the ATM gene into this cell line resulted in an increased resistance to oxidative stress (Figure 4). Since a significant aspect of the AT cellular phenotype is an increased susceptibility to oxidative stress, the data presented here indicate that viral transduction of the ATM sequence results in phenotypic reversal more similar to that of a normal cell. Thus, viral transduction of ATM results in ATM gene expression, and some correction of the pathological phenotype of the AT cell.

Neurodegeneration is one of the main pathological changes in individuals with AT, with severe cerebellar degeneration being common. In individuals with AT, cerebellar Purkinje cells are also found to have abnormal patterns of dendritic arborization, and are displaced in the monolayer of the cerebellar cortex.[4,46] In mice, Western blot analysis has previously demonstrated the presence of ATM protein in a wide variety of tissues, including the brain.[47] Northern blot analyses of RNA isolated from a number of different human brain regions, including cerebellum, cerebellar cortex, cerebellar medulla, spinal cord, frontal lobe, temporal lobe, occipital lobe and putamen, demonstrated an expected 12 kb AT transcript with relatively uniform distribution. While the RNA levels for ATM in most brain regions are similar, the frontal and temporal lobes show low to absent levels. Northern blot analyses of ATM expression of mouse brain RNA revealed similar levels and patterns as man.[48] Oka and Takashima investigated the expression of the ATM protein in the human brain.[49] By immunohistochemistry, the ATM protein was detected in the cerebellar cortex, but not in the cerebral cortex (the frontal lobe), at the late gestational stage.[49] Cortical neurons in the frontal lobe temporarily exhibited immunoreactivity at the early gestational period of humans. Purkinje cells exhibited the most intense immunoreactivity in AT during late prenatal and early postnatal periods, followed by persistent and moderate reactivity in the adult samples. Granular cells only showed a temporary staining during the gestational period.[49]

We report here, for the first time, the distribution of ATM protein immunoreactive neurons in the forebrain and cerebellum in adult rats (Figure 5). We observed strong AT staining in the frontal, parietal, temporal cortex, and the piriform cortex, and weak AT staining in the caudate-putamen, the globus pallidus, and the claustrum. In the cerebellum, AT-stained neurons were found in the Purkinje cell layer and the monolayer, but not in the granular cell layer, or the cerebellar cortex. In addition, AT-stained cells were also found in the cerebellar nuclei. We noticed that ATM was predominantly located in the nuclei, but in some cells, such as the Purkinje cells, cells of the cerebellar nuclei, and ventral horn neurons of the spinal cord, ATM protein was also found in the cytoplasm (data not shown). This is consistent with the observation of endogenous ATM protein in normal fibroblast cells detected with immunofluorescent histochemistry.[7] A prior report emphasized that the ATM was distributed within the cytoplasm of the Purkinje cells, but not within the nuclei.[49] This discrepancy may stem from the differences within species or from different techniques used to stain the cells. Further studies will be required to more precisely delineate these differences.

Although not currently possible, the direct delivery of ATM genes into large areas of the brain is future hope for AT gene therapy. As a much more modest step, we here document ATM gene delivery into some cells within the rat brain (Figure 6). After our successful confirmation of ATM gene expression *in vitro*, the pTO-ATM viral vector was injected directly into the rat caudate-putamen, which originally showed little or no staining of endogenous ATM protein. We decreased the background staining from endogenous ATM protein by using thinner brain sections (10 μm rather than 30 μm). This technique was performed in the experiments mapping ATM protein in the forebrain and the cerebellum. This technique also allowed us to see enhanced expression of the ATM gene delivered in the rat caudate-putamen after *in vivo* gene transfer by direct injection of the pTO-ATM vector. Noticeably, tissue damage is visible around the site of injection (Figure 6). It is apparently due to the physical damage of the injection needle.[33] No staining was observed in the caudate-putamen in the contralateral injection site. It is also believed that the HSV helper virus will introduce cytotoxicity to transduced cells.[50–52] Using less cytotoxic helper viruses by deletion of more IE genes[45,53–58] or using helper virus-free packaging systems as an attempt to reduce cytotoxicity and immunogenicity[59–63] would be a future solution. No staining in the injection site was observed after PBS was substituted for the primary antibody. The ATM expression lasted for at least 7 days after direct injection. Longer-term expression was not observed and remains a major challenge for gene therapy research to treat AT. Our future plans are to employ a tissue specific or an endogenous promoter to control AT gene expression. If higher levels of gene

delivery can be obtained, attempts to functionally correct the defect of Atm mice may be considered. Various behavioral tests could be performed after gene delivery into the cerebellum region of the mice. The data presented here provide preliminary groundwork for further development of gene therapy for AT.

Materials and methods

Cell culture

AT5BIVA, an SV40-transformed AT cell line, was obtained from Coriell Cell Repositories (Camden, NJ, USA). The positive control cell line VA13, an SV40-transformed normal human fetal lung fibroblast cell line, was obtained from ATCC (Rockville, MD, USA). MRC-5 cell is a primary cultured normal cell line and was obtained from Dr Paules, NIEHS. AT22IJE-T (AT22) is an SV40-transformed AT cell line and AT22IJE-T PEBS7-YZ5 (YZ5) cell is an AT22 cell line transduced with ATM gene. Both cell lines were obtained from Dr Shiloh, Tel Aviv University, Israel. The 2–2 helper cell line (kindly provided by Dr Sandri-Goldin, University of California, Irvine) containing the HSV IE2 gene was derived from Vero cells, and was used for packaging of amplicons. All cells were grown in Dulbecco's modified Eagles medium (DMEM, Invitrogen, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin/glutamine (Invitrogen). The MRC5 cells were grown under similar conditions with 20% FBS. The YZ5 cells were grown with hygromycin at 100 μg/ml.

Construction of pTO-ATM amplicon vector

The HSV amplicon vector pTO11, kindly provided by Dr N Stow, MRC, Glasgow, UK, contains the **a** packaging signal and the ori_s of HSV-1. The pTO11 was digested with *Nsp*I and self-ligated to generate pTO-*Nsp*I. The pTO-*Nsp*I vector was then digested by *Hind*III and *Bam*HI, and ligated with an *Mlu*I and *Bam*HI fragment from phGFP-S65T vector containing a humanized, red-shifted GFP gene, a CMV immediate-early promoter and a poly A signal. This plasmid was named pTO-GFP. The pTO-GFP was further digested with *Xba*I and *Hind*III, and the GFP gene was replaced by a 9.4 kb *Sal*I and *Xho*I fragment containing a 9.168 kb ATM ORF. This second construct, pTO-ATM, was also driven by the CMV immediate-early promoter (Figure 1).

Viral vector packaging

2–2 cells[64] (1.5×10^6/well) were cultured in a 100 mm plate (Corning Incorporated, Corning, NY, USA). The pTO-ATM plasmid was transfected into 2–2 cells with NovaFector by the manufacturer's protocol (VennNova, LLC). After 24 h, the cells were infected with 0.5–1.0 MOI of 5dl1.2 virus (HSV-1 helper virus with a deletion of the IE2 gene, kindly provided by Dr P Schaffer, Harvard University)[65] in 0.5 ml of the packaging medium (DMEM plus 6% FBS, 4 mM L-glutamine). Infection continued for 2 h at 37°C in a humidified, 5% CO_2 incubator. Then, 1.5 ml of packaging medium was added and cells were incubated for an additional 44–48 h. By then, the cells showed cytopathic effects (CPEs) and were harvested by scraping the cells and the culture medium into conical tubes. After the initial centrifugation at 605 g, cell debris and supernatant were separated, and the pelted portion was resuspended and subjected to three freeze/thaw cycles to release the viral vectors. The remaining debris was cleared by centrifugation at 2400 *g*. For purification, viral vectors were filtered through disposable Nalgene 0.8 μm nylon filter bottles (Nalgene, Rochester, NY, USA). For concentration, this filtered viral supernatant was put into Beckman polyallomer Quick-Seal centrifuged tubes (Beckman, Palo Alto, CA, USA). The tubes were then ultracentrifuged at 75 000 *g* at 4°C in a Beckman 55.2 Ti rotor for 1 h to pellet the virions. The viral pellet was resuspended with sterile Hank's Balanced Salt Solution (HBSS, Invitrogen) buffer for further application.

Vector titration

For pTO-ATM amplicon titration, 2.5×10^5 AT5BIVA cells per well were plated in 24-well plates. The next day, the wells containing cells were transduced with various dilutions of pTO-ATM vector stock in Opti-MEM media (Invitrogen). After 2 h, 0.8 ml of DMEM was added to each well. After 24 h incubation, the cells were fixed and stained by immunohistochemistry (see method below). In all, 10 optical fields per well were counted for ATM-positive staining cells under a ×20 objective. The total number of positive cells per well was then calculated and the titer of the pTO-ATM viral stock was determined accordingly. For helper virus titration, the pTO-ATM viral stock was serially diluted in Opti-MEM medium and placed onto a confluent 2–2 cells monolayer in six-well plates. After 2 h of incubation with rocking every 20 min, the virus solution was aspirated, cells were washed with HBSS, overlaid with 50%, 2× DMEM with 10% FBS and 50% of a 0.6% methylcellulose solution, and incubated for 3 days. The cells were fixed and the plaques were visualized by staining with 0.1% crystal violet for 10 min. The plaques were counted and helper virus titers were calculated.

Western blot

Cells were plated in 100 mm tissue culture dishes and infected 24 h later with pTO-ATM or pTO-GFP vectors at an MOI of 0.01. At 48 h after infection, cells were washed with 1× PBS three times and pelleted. The pellet was resuspended with 250 μl of 1× lysis buffer (1% NP-40, 10 mM sodium phosphate (dibasic), 1 mM EDTA, 5 mM β-glycerophosphate, 1 mM DTT, and 150 mM NaCl), with protease inhibitors (Aprotinin 3.995 μg/ml, Leupeptin 0.75 μg/ml), and this suspension was placed in a microcentrifuge tube. The suspension was centrifuged at 4°C for 20 min at 13 000 *g* and the supernatant, total cellular protein, was put into a microcentrifuge tube for protein quantification or storage at −70°C. Primary antibody N514 (kindly provided by Dr Paules) was added to each protein sample and tumbled at 4°C for 2 h. Protein G-sepharose beads were added with a cut tip and tumbled for an additional 30 min. Following quick spins at 10 450 g for ∼5 s, the soft pellet was washed 3× with ∼1 ml of lysis buffer. A measure of 5× loading buffer was then added to each sample before boiling for 5 min. Samples were next centrifuged at 13 620 *g* for 5 min and pipetted from the beads into clean microcentrifuge tubes that were stored for up to 3 days at room temperature. Samples were then boiled and loaded onto a Novex 3–8% gradient gel, as per the manufacturer's

suggested protocol. The gel was run for ~1 1/2 h at 150 V. The gel was then transferred at 90 V for 7 h, at which time it was turned down to 35 V overnight. The blot was then rinsed briefly with 1 × TBST (20 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) and then the nitrocellulose was blocked with 5% milk in 1 × TBST for 3 h. Another brief rinse was performed with 1 × TBST and then Gene Tex ATM-2C1 was added at 1:1000 dilution for 1 h. The blot was washed 3 × for 10 min with 1 × TBST. Amersham NA931 was then added for 1 h, 1:4000. The blot was washed three times for 10 min each with 1× TBST, after which it was developed using the Roche ECL kit.

Colony-forming assay

Colony-forming-efficiency experiments were carried out as described previously.[66] In brief, cells were transduced with pTO-ATM or pTO-GFP vectors at an MOI of 0.1. After 12 h, the transduced cells were plated at 4000 cells/100 mm tissue culture dish in 10 ml media (DMEM, 5% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin), allowed to adhere for 18 h, and treated with a 15 min exposure to 10 μM *t*-butyl hydroperoxide. The cells were allowed to adhere for 18 h, as this period allowed maximal time for cellular adherence, but was too short for the cells to enter mitosis and thus invalidate experimental reliability. Following *t*-butyl hydroperoxide exposure, the plates were washed 2 × with HBSS, the media replaced, and, after 14 days, the colonies were fixed and stained with methanol, followed by a solution of crystal violet (1 g/l). The colonies were then counted under a dissecting microscope. Survival was calculated as a percentage of untreated cells. 'Colonies' consisted of cell clusters containing 50 or more cells. The AT22 cell line was used at passages 20–30, the MRC5 cells between passages 18 and 21, and the YZ5 cells at passages 23–25. All experiments were performed in triplicate and repeated at least twice. Standard deviations (error bars) were calculated based on the average untreated value. The mean standard deviation was determined by averaging the standard deviation between experiments.

Immunohistochemical detection of ATM gene product in vitro

AT5BIVA cells (2.5×10^5 per well) were plated in 18 wells of a 24-well plate. Six wells of the same 24-well plate were plated with 2.5×10^5 VA13 cells used as a positive control. After 24 h, 12 wells of AT5BIVA cells were infected with 0.005–0.5 MOI of pTO-ATM viral stock solution, and the remaining six wells of AT5BIVA cells were exposed to HBSS solution as negative controls. At 24 h after infection, all cells were fixed with 4% paraformaldehyde in 1 × PBS (pH 7.2) at 4°C for 30 min. The cells were incubated with goat anti-ATM primary antibodies (1:100; Atm, Q-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 0.3% Triton X-100 and 10% normal horse serum in PBS for 2 h at room temperature and then at 4°C overnight. The cells were incubated subsequently with rabbit anti-goat secondary antibody (Sigma, 1:100) conjugated with alkaline phosphatase in 10% normal horse serum in PBS at room temperature for 2 h. The color reaction was done with a BCIP/NBT substrate tablet (Sigma) dissolved in distilled water. Several washes with 1× PBS were done at the intervals between and after incubations.

Immunohistochemical detection of ATM transgene product in the rat brain

Male Sprague–Dawley rats (250–300 g; Harlan, Indianapolis, IN, USA) were used. All animals were cared for under an approved animal protocol, using the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) guidelines. To determine the normal distribution of ATM protein in the rat forebrain and cerebellum, five rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (8 mg/kg), and then perfused through the ascending aorta with saline, followed by cold 4% paraformaldehyde in PBS (pH 7.4). The brains were subsequently removed, postfixed in the same fixative for 4–6 h and immersed in 25% sucrose in PBS (pH 7.4) at 4°C. The forebrain and the cerebellum were serially cryosectioned at 30 μm thickness with a cryostat. The sections were collected in PBS (pH 7.4). Free-floating brain sections were incubated with goat anti-ATM primary antibodies (Atm, Q-19, Santa Cruz Biotechnology; 1:300) in 0.3% Triton X-100 and 10% normal horse serum in PBS for 2 h at room temperature, and then at 4°C overnight. The sections were incubated subsequently with biotinylated mouse monoclonal anti-goat secondary antibody (Sigma, 1:200) in 10% normal horse serum in PBS at room temperature for 2 h. The third incubation was performed with avidin-biotinylated horseradish peroxidase (Sigma, 1:200) in PBS. The color reaction was performed by using diaminobenzidine peroxidase substrate tablet (Sigma) in the presence of 0.02% H_2O_2 in 0.05 M Tris-buffered saline (pH 7.6). Several washes with PBS were done at intervals between and after incubations. The immunostained sections were mounted on polylysine-coated glass slides, dehydrated, cleared and covered with Cytoseal 60 Mounting Medium (Stephens Scientific, MI, USA). Neuroanatomic structures were identified according to the rat brain atlas by Paxinos and Watson.[67]

Stereotaxical injection of pTO-ATM viral vector into the rat brain

In all, 15 rats were anesthetized with ketamine/xylazine as previously described.[33] A volume of 4 μl of pTO-ATM viral stock solution (amplicon titer: 8.7×10^7 infection units/ml) was stereotaxically injected into one side of the caudate-putamen of each rat. The following stereotaxic coordinates for the middle of unilateral caudate-putamen were used: AP, 0.5; ML, 3.0 and DV, 5.5. On days 4, 8, and 14, five animals were anesthetized again with ketamine/xylazine and then perfused as above. The brains were postfixed and cryoprotected. The whole brain was serially cryosectioned at 10 μm thickness. The sections were directly attached on polylysine-coated slides (Sigma). The same immunohistochemical procedure using the secondary antibody conjugated with alkaline phosphatase, as noted above, was employed on these sections to detect the expression product of ATM transgene in the rat caudate-putamen. The immunostained sections were covered with Crystal/Mount mounting medium (Biomeda Corp., Foster City, CA, USA). To test the specificity of the anti-ATM antibody, we

absorbed the antibody with five-fold blocking peptide (Santa Cruz) or substituted the antibody with PBS, and then used this solution to incubate the brain sections and perform immunohistochemistry.

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