Adeno-associated virus type 2 interferes with early development of mouse embryos

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The human helper-dependent adeno-associated virus type 2 (AAV-2) has been shown to induce differentiation in various cell types in culture including pluripotent embryonic cells, in the absence of helper virus. To assess whether induction of differentiation may influence developmental processes we analysed the effect of AAV-2 on developing mouse embryos. In vitro infection of fertilized eggs induced arrest of development at the two-cell stage. Moreover, micro injection of AAV-2 DNA (comprising either the complete AAV-2 genome or a fragment containing the P5 promoter region) into one-cell embryos, blocked development at the morula stage. In vivo, AAV-2 infection of pregnant mice led to fetal death and early abortion. These results demonstrate that the human adeno-associated virus, which is thought to be non-pathogenic, can perturb embryonic development in mice. This may provide a suitable animal model system to further elucidate the biological significance of the recent detection of adeno-associated virus DNA in human abortion material.

Introduction

Adeno-associated viruses (AAV, types 1 to 5) are members of the family of Parvoviridae comprising small (about 20 nm diameter), icosahedral, non-enveloped viruses with a ssDNA genome of approximately 5000 nucleotides characterized by identical palindromic termini of 145 bases (Siegl et al., 1985). The left part of the AAV genome encodes four overlapping non-structural proteins (rep) which are translated from differentially spliced mRNAs driven by the P5 and P19 promoters. In the right part of the genome, three overlapping capsid polypeptides (VP proteins) are encoded from the P40 promoter (Berns & Bohensky, 1987). For replication, AAVs strongly depend on host cell functions and, usually, on co-infection by helper viruses such as adenovirus, herpesvirus or vaccinia virus (Berns & Bohensky, 1987; Buller et al., 1981; Schlehofer et al., 1986). In certain cell systems, treatment with chemical or physical carcinogens can support low levels of AAV replication in the absence of a helper virus (Schlehofer et al., 1983; Yakobson et al., 1987; Yalkinoglu et al., 1988).

Without helper virus co-infection, DNA of infecting AAV frequently integrates into the host cell genome at preferential chromosomal loci (Kotin et al., 1990; Walz & Schlehofer, 1992), a process which is mediated by the terminal repeat sequences of AAV DNA (Samulski, 1993). The viral genome may remain in this latent phase for many cell generations, but retains its ability to be rescued and replicated when helper viruses infect the cells (Kotin et al., 1990; Laughlin et al., 1986; Walz & Schlehofer, 1992).

AAV has not been found to be associated with disease in humans, nor in laboratory animals (Siegl et al., 1985), in contrast to autonomous parvoviruses some of which are the cause of fetal disorders in humans, [parvovirus B19 (Anderson & Hurwitz, 1988; Kinney et al., 1988; Schwarz et al., 1988; Torok, 1990; Woernle et al., 1987)] and animals [e.g. porcine parvovirus and minute virus of canines (Carmichael et al., 1991; Joo et al., 1976, 1977)]. For AAV type 1 (AAV-1) a transplacental route of transmission in mice has been reported (Lipps & Mayor, 1980; Lipps & Mayor, 1982).

Similar to members of the autonomous parvovirus group (e.g. H-1), AAV has been shown to display onco-suppressive activity in vitro and in vivo (for reviews see: Rommelaere & Cornelis, 1991; Schlehofer, 1994). Recent results from our laboratory have shown that human keratinocytes (HaCat) and leukaemia cells (HL60) can

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be induced to differentiate by infection with AAV-2 (Klein-Bauernschmitt et al., 1992). This may be involved in the tumour suppressive activity of these viruses. Since induction of differentiation-associated antigens by AAV-2 has recently been demonstrated in murine embryonic stem cells as well (V. Botquin & J. R. Schlehofer, unpublished) we considered whether induction of differentiation may interfere with developmental processes. Therefore we analysed the influence of AAV-2 on mouse embryo development. Here we report (i) that infection with AAV-2 or micro injection of AAV-2 DNA into fertilized eggs led to arrest of development and (ii) that early abortion was induced in pregnant mice after infection with AAV-2. Since DNA sequences containing only the AAV-2 early promoter (P5) region were sufficient to induce growth arrest of embryos it appears that viral gene expression is not required for this effect.

Methods

Virus. AAV-2 was propagated in HeLa cells using adenovirus type 2 (Ad2) as a helper. When Ad2-induced c.p.e. was complete, cells were lysed by freezing and thawing and then were centrifuged in a SS-34 rotor (Sorvall; Du Pont) at 22000 r.p.m. for 3 h. Virus was extracted from the pellets by the deoxycholate–trypsin method (Rose et al., 1969), and AAV-2 virions were separated from Ad2 virions by equilibrium centrifugation through a CsCl density gradient, as described previously (de la Maza & Carter, 1980). To obtain infectious AAV-2, the band of 1.41 g/cm³ was collected (adenovirus particles band at 1.35 g/cm³). Finally, AAV-2 was dialysed against a solution containing 0.3 M-NaCl and 50 mM-Tris-HCl pH 8.0 and heated to 56 °C for 30 min to inactivate any residual Ad2 contamination. The titre of purified AAV-2 was determined by dot-blotting of AAV- plus Ad2-infected HeLa cell cultures on nylon membranes (Gene-Screen; Du Pont-NEN) hybridized with radiolabelled AAV-2 DNA as described elsewhere (Bantel-Schaal & zur Hausen, 1988). One-cell embryos were infected by placing them in micro drops of M16 medium containing purified AAV-2. The in vitro development was observed for the periods of time indicated in the respective figures. Controls were done using ‘mock infection’ a dialysed fraction of 1.41 g/cm³ collected from a CsCl density gradient containing only (in addition heat-inactivated) Ad2. Pregnant mice were intravenously infected by injecting 100 μl containing 10⁵ to 10⁶ purified virus suspension (in 0.9 % NaCl) in the vein of the tail (for m.o.i. and times of gestation see Results).

Collection and culture of mouse embryos. Gonadotropins were administered to female mice ([C57 BL/6 × C3H] F1 hybrids, supplied by the Zentralinstitut für Versuchstierkunde, Hannover, Germany) prior to mating in order to increase the number of ovulated eggs. Pregnant mare’s serum (5 infectious units (IU) PMS, Sigma) was injected intraperitoneally into females followed 48 h later by 5 IU of human chorionic gonadotropin (hCG, Sigma). Females were mated with (C57 BL/6 × C3H) F1 hybrid males. The next morning, the females were checked for a copulation plug to confirm mating (day 1 of gestation). Fertilized mouse eggs used for micro injection or infection were collected from pregnant mice at day 1 of gestation. Female mice were killed by cervical dislocation. The oviducts were transferred to M2 medium containing hyaluronidase (300 μg/ml) as described elsewhere (Hogan et al., 1986). The fertilized eggs were picked up from the ampulla and transferred to fresh micro drops of M16 medium (Hogan et al., 1986) and then were fixed in paraformaldehyde. The embryos were incubated at 37 °C in an atmosphere containing air with 5% CO₂. One-cell embryos developed into blastocysts within 5 days of culture.

AAV-2 DNA constructs. The cloned AAV-2 genome (pTAV2 (Heilbronn et al., 1990)) were cut from the pBluescript II KS vector by digestion with Pvu II, separated using agarose gel electrophoresis and purified using the Gene Clean kit (Dianova). A plasmid containing the terminal repeat sequences of AAV-2 and the early promoter region (P5 promoter) was obtained by cutting pTAV2 with Neo I which has three restriction sites in AAV-2 (at the nucleotide positions 625, 3767 and 4488). The internal portion of the AAV-2 genome was excised (from nucleotide 625 to 4488), the terminal fragments were ligated thereby generating the plasmid pAV2TR. Another AAV-2 DNA sequence (fragment P5) of 160 bases containing the P5 promoter region (from nucleotide 190 to 350) was obtained by digestion of pAV2TR with Ava II. To obtain a fragment representing only one terminal repeat (called 1TR), we digested the plasmid pAV2TR with Pvu II (cutting at nucleotide position 529 of pBluescript) and Ava II (at positions 190 of AAV-2). Prior to micro injection, the AAV-2 DNA fragments were separated from the pBluescript II KS vector, purified as described above and resuspended in injection buffer (5 mM-Tris pH 7.4/0.1 mM-EDTA). The different constructs of AAV-2 DNA were introduced by micro injection into the pronuclei of (C57 BL/6 × C3H) F1 mouse eggs as described elsewhere (Hogan et al., 1986).

Immunofluorescence analysis. Embryos were fixed for 5 min in pre-cooled methanol (–20 °C) and were pipetted onto cover-slips. After washing in PBS, the embryos were permeabilized for 4 min in 0.25 % Triton X-100, then washed three times in PBS and stained with the first antibody [monoclonal mouse anti-rep (anti-AAV-2 early proteins) or polyclonal rabbit anti-VP (anti-AAV-2 capsid proteins)] for 1 h at room temperature. After washing twice with PBS, the coverslips were incubated for 1 h with fluorescein anti-mouse (against rep) and fluorescein anti-rabbit (against VP) IgG (Dianova). The coverslips were then washed six times and mounted with 90 % glycerol and 10 % PBS. Immunofluorescence was analysed using a Leitz (Wetzlar) fluorescence microscope.

Results

In vitro infection of mouse embryos with AAV-2

To assess whether AAV-2 infection interferes with embryo development, we collected fertilized oocytes from superovulated female mice at day 1 of gestation and infected them in vitro with different amounts of AAV-2 (from 10⁴ to 10⁷ infectious units in 30 μl micro drops cultures; the virus was heat-inactivated to avoid effects of any residual infectious adenovirus (used as a helper for AAV-2 propagation) eventually present despite CsCl purification of AAV-2, (see Methods). Mock-infected (control) embryos were incubated with a dialysed fraction of 1.41 g/cm³ [i.e. the AAV-2 containing fraction in adeno+AAV-2 preparations (adenovirus particles band at 1.35 g/cm³)] collected from a CsCl density gradient containing only (heat-inactivated) adenovirus type 2. The eggs were incubated at 37 °C and 5% CO₂ and development in vitro was then monitored for 5 days (until the embryos reached the blastocyst stage). Indirect immunofluorescence revealed the binding of viral par-
AAV-2 mediated arrest of embryo development

Fig. 1. In vitro development of embryos infected with different doses of AAV-2. One-cell embryos were infected with the indicated amounts (IU) of AAV-2 virus in 30 μl micro drops culture. Embryo development was followed for 5 days. The effect of AAV-2 on embryo development at days 2 and 3 after infection and uninfected control (C) are shown in (a) and (b), respectively.

(a) At day 2, 80% of mock-infected embryos (control) had developed to the two-cell stage as had all AAV-2-infected embryos and this was independent of the infectious dose. (b) At day 3, the development of infected embryos was found to be blocked at the two-cell stage or embryos were dead (85% with 10^8 AAV-2 infectious units). With 10^7 or 10^6 infectious units, 40% of the embryos arrested at the two-cell stage or were dead. (c) Shows the morphology of embryos infected with different doses of AAV-2 (10^8, 10^7 and 10^6 IU) in comparison with mock-infected embryos (3 days post-infection). Scale bar represents 100 μm.

Micro injection of AAV-2 DNA into fertilized mouse oocytes

To analyse whether AAV-2 DNA may interfere with embryo development in vitro, we micro injected AAV-2 DNA (complete genome), or vector (pBluescript) sequences as a control, into one-cell embryos which were then transferred into droplets of M16 medium. Development was observed for 5 days. Ninety percent of control (Bluescript) embryos developed normally into morulas, as did embryos injected with a LacZ gene [expressed from a human papillomavirus gene promoter as described previously (Cid et al., 1993)]. In contrast, approximately 85% of embryos micro injected with AAV-2 DNA were blocked at the morula stage and died.
construct pAV2TR contains the P5 promoter and hence the MLTF element, we isolated a 160-bp fragment comprising the P5 promoter region devoid of terminal repeat sequences for micro injection into one-cell embryos. With this DNA fragment (fragment P5 in Fig. 4b), an average of 60% of the embryos were blocked at the morula stage.

**AAV-2 induced early abortion in pregnant mice**

In order to determine *in vivo* the influence of AAV-2 on embryo development, we infected pregnant mice (intravenously, on the first day of gestation) with different doses of AAV-2 (10⁶, 10⁷, 10⁸, 10⁹ and 10¹⁰ IU in 0.1 ml; conducting four independent experiments using a total of 20 animals). Infected and control animals (mock-infected as described above) were analysed at the termination of pregnancy (19 days). Mice infected with 10⁵ to 10⁸ IU of AAV-2 did not deliver offspring, indicating that abortion had occurred. Histologically, signs of fetal death with intrauterine resorption were observed (data not shown). Infection with 10⁴ infectious particles of AAV-2 did not impair development of the embryos, and the progeny of these mice were completely normal.

In order to determine which stages of development were sensitive to AAV-2 infection, pregnant mice (20 animals) were intravenously infected with AAV-2 (10⁸ particles) at day 1, 3, 5, 7 and 10 of gestation. AAV-2 infection inhibited embryo development until day 7 of gestation. At later stages of pregnancy the development of embryos was not sensitive to AAV-2 infection and normal offspring were observed in all cases.

Using PCR analysis, AAV-2 DNA was detectable (one week after infection) in many tissues of AAV-2-infected pregnant mice, including the uterus, whereas in non-pregnant mice uterus tissues did not contain AAV DNA (Botquin, 1994).

**Discussion**

Previous results from our laboratory had shown that infection with AAV-2 induced differentiation in established cell lines (Klein-Bauernschmitt *et al.*, 1992) and in pluripotent embryonic cells (Botquin, 1994; V. Botquin & J. R. Schlehofer, unpublished).

It appeared important to assess whether effects on differentiation in *in vitro* systems extend to developmental processes in mice, notably in view of the fact that infection with other paroviruses during pregnancy has been associated with fetal disease, stillbirth and abortion (Brown *et al.*, 1984; Carmichael *et al.*, 1991; Forman *et al.*, 1977; Hogg *et al.*, 1977; Joo *et al.*, 1976, 1977). In order to follow embryonic development after AAV-2 infection, we performed *in vitro* infection of fertilized...
AAV-mediated arrest of embryo development

Growth arrest at the two-cell stage was observed with high doses of AAV-2 virions. Apparently, no expression of AAV-2 early proteins (rep) was needed to accomplish this effect. Similarly, micro injected AAV-2 DNA was able to interfere with embryonic development. After micro injection of the complete genome of AAV-2 into fertilized oocytes, more than 80% of embryos containing AAV-2 DNA were blocked at the morula stage. Under these conditions, AAV rep proteins were shown to be expressed during the first three days of development, until embryo death. However, arrest of development was also observed after micro injection of non-coding sequences of AAV-2 excluding the possibility of rep gene expression. Therefore, rep proteins do not seem to play an essential role in the observed effects. It is known that the early non-structural proteins (NS) of autonomous paroviruses are cytotoxic (reviewed in Rommelaere & Cornelis, 1991). Expression of the non-structural rep proteins of AAV-2 (corresponding to the NS proteins) may exert some cytotoxic effects and contribute to the higher rate of growth arrest when the whole AAV-2 genome was micro injected. The assumption that expression of AAV genes after infection is not necessary for the observed effects is supported by the observation that u.v.-inactivated AAV-2 particles also have the potential to induce differentiation in established cell lines (P. Klein-Bauernschmitt & J. R. Schlehofer, unpublished) and also cause early abortion in pregnant mice (Botquin, 1994). This suggests that the effects of AAV on development do not require viral gene expression and may be associated with the viral DNA. The hypothesis of a role of AAV-2 DNA is supported by our finding that micro injection of non-coding fragments of AAV-2 DNA into fertilized oocytes led to an early arrest of embryonic development. Since integration of AAV-2 has been reported to modify the biological properties of cells (Walz & Schlehofer, 1992; Winocour et al., 1992) a possible mechanism for disturbance of embryonic growth might be the integration of AAV-2 sequences into gene(s) critical for development.

Another possibility may be an interaction of cellular regulatory factors with AAV-2 DNA leading to inappropriate binding or a competition for factors important in embryonic development. A lethal effect of DNA on embryo development has been reported recently.

Fig. 3. Growth arrest of embryos developed from fertilised eggs micro injected with the complete genome of AAV-2 or the two terminal repeat sequences [containing also one promoter region (P5) of AAV-2 (construct pAV2TR)]. Development was followed for 5 days. ■, Percentage of blastocysts; □, percentage of dysplastic morulas. (a) shows that 85% of embryos micro injected with the complete genome of AAV-2 (day 5), and 75% of embryos injected with the terminal repeat sequences of AAV-2 arrested at the morula stage. In addition, 90% of embryos micro injected with Bluescript (control) were developed normally to blastocysts. These data represent results from four independent micro injection experiments in which approximately 250 eggs were micro injected. (b to d) Morphology of embryos micro injected with the AAV-2 complete genome (c), the plasmid pAV2TR (d) or Bluescript (control, b). The majority of injected embryos arrested at the morula stage, whereas embryos micro injected with Bluescript developed to blastocysts. Scale bar represents 8 μm.
Another high identity (80%) was found between the terminal repeat sequences of AAV-2 DNA and a region of the mouse retinoic acid responsive gene (Matsubara et al., 1990). The region of high identity is found in one exon corresponding to the major retinoic acid responsive gene transcribed mRNA. This gene is intensely expressed at early differentiation stages of embryonic carcinoma cells and in a defined gestation period of mouse embryogenesis (Muramatsu, 1993). It remains to be determined if these identities have any relevance for the effects on development observed with micro injected AAV-2 DNA. Studies are under way to determine in detail the sequence(s) of AAV-2 DNA which is (are) necessary to mediate arrest of embryo development.

In previous attempts to obtain transgenic mice using the complete AAV-2 genome, we failed to detect transgenic-positive offspring. It is tempting to speculate that this was possibly due to the effects of AAV-2 DNA on embryonic cells described herein.

In the initial experiments to assess a possible influence of AAV-2 on development in vivo, we observed early abortion (intrauterine fetal death) in pregnant mice infected with high doses of AAV-2. An AAV-2-associated termination of pregnancy could be found up to the seventh day of gestation. Previous work in experimental teratology attempting to determine the particularly sensitive stages in pregnancy indicated that the most sensitive period of intrauterine development, encompassing germ layer formation and organogenesis, corresponded to the first seven days of mouse embryo genesis (Müller & Streffer, 1990).

The in vivo data described here do not yet provide information on the mechanisms by which embryo development in pregnant mice may be impaired by AAV-2 infection. It remains to be determined whether the target tissue of AAV-2 infection in vivo is the developing embryo itself or whether placenta or uterus tissues are disturbed by the virus. In addition, we cannot exclude that maternal factors induced after intravenous infection are involved in pregnancy termination. This possibility is favoured by the striking observation that induction of abortion is mediated by relatively low dose of virus in contrast to the high titres required for arresting embryo development in vitro. However, it is interesting to note that in pregnant or superovulated mice, the uterus appears to be a rather specific target tissue for intravenously injected AAV-2 whereas in control mice the virus was not detected (using PCR analysis) in uterus tissue (Botquin, 1994). Furthermore, we cannot exclude that the mechanisms whereby AAV-2 infection interferes with the development of embryonated eggs in vitro are different from the in vivo situation. In the early stages of pregnancy, combined mechanisms may play a role, involving (premature?) differentiation of embryonic...
tissue (embryo or placenta) by binding of viral particles to the cellular membrane (P. Klein-Bauernschmitt & J. R. Schlehofer, unpublished; Winocour et al., 1988), effects of parvoviral DNA, and maternal factors.

These issues require further detailed analyses. The high amount of AAV DNA molecules micro injected, and the high multiplicity of infectious particles used in these infection experiments may not resemble the conditions that occur in nature. However, these conditions have allowed us to make certain observations on the effects of AAV-2 on development. Therefore, this study may assist towards establishing a mouse model system for studying the significance of the recent detection of AAV-2 DNA incurrageation material from spontaneous abortion in humans (Tobiasch et al., 1994).

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