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Journal of Medical Virology

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Journal:	*Journal of Medical Virology*
Manuscript ID:	JMV-09-1419.R3
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	11-Mar-2010
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Keywords:	adenovirus , histologic chorioamnionitis, placenta, preterm birth , viral infection

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Short title: Adenovirus in the placenta and preterm birth

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Adenovirus is isolated frequently from the amniotic fluid and has been implicated in severe neonatal infections. A case control study was carried out to examine the association of detection of adenovirus in placentas with preterm birth and histological chorioamnionitis. Placentas from preterm and full term deliveries were collected prospectively. Preterm cases were divided into three subgroups according to the gestational age. PCR was carried out on placental tissues for the detection of adenovirus genome. Placentas were evaluated histologically for the presence of chorioamnionitis. Chi-square and odds ratios (OR) were used to determine if detection of adenovirus is associated with preterm birth and histological evidence of inflammation. Seventy-one preterm and 122 full term placentas were studied. Adenovirus genome was detected in 29 (40.8%) of preterm cases and in 25 (20.5%) of the full term controls (OR =2.6; 95% CI, 1.4-5.1; *P*=0.002). Detection of adenovirus in preterm placentas was significantly higher compared to full term particularly in the lower gestational age. Detection of adenovirus in placenta followed the seasonal variation of adenovirus infections. Thirty-seven preterm and 21 full term placentas were also selected for paraffin inclusion and histological examination. Chorioamnionitis was present more frequently in preterm adenovirus-positive placentas compared to preterm adenovirus-negative placentas (75% vs 36%; *P*=0.03) as well as compared to term adenovirus-positive placentas (75% vs 19%; *P*=0.003). This study demonstrates that adenovirus infection of the placenta is associated strongly with histological chorioamnionitis and preterm birth.

Keywords: adenovirus; histological chorioamnionitis; placenta; preterm birth; viral infection

INTRODUCTION

Since Knox and Hoerner [1950] first recorded an association between infection and preterm birth, there has been extensive evidence associating inflammation and infection to spontaneous preterm birth, especially when this occurs at less than 28 weeks of gestation [Goldenberg et al., 2003; Iams, 2003, Steer, 2005; Romero et al., 2007]. Various bacterial and viral agents have been linked to the pathogenesis of preterm birth. Bacteria recovered from the amniotic cavity following preterm delivery include *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, group B *Streptococcus*, *Bacteroides* spp., and *Trichomonas vaginalis* [Goldenberg et al., 2003; Romero et al., 2007]. Despite the fact that viral infection during pregnancy may prove to be devastating, evidence based etiology on the role which specific viral infections may play in preterm birth still remains rare. A particularly interesting finding is that adenovirus, a double-stranded DNA virus associated commonly with conjunctivitis and upper respiratory tract infections, is detected more frequently in amniotic fluid samples in comparison to other viruses such as cytomegalovirus, herpes simplex virus, enteroviruses and parvovirus [Van den Veyer et al., 1998; Baschat et al., 2003a]. Congenital adenovirus infections have indeed been associated with a variety of adverse effects on the fetus ranging from complications such as pleural effusion, echogenic liver foci to more severe complications including myocarditis and central nervous system anomalies [Meyer et al., 1985; Baschat et al., 2003b].

In several case reports intrauterine adenovirus infections in preterm and full term neonates have been reported to result in a fatal outcome [Towbin et al., 1994; Montone et al., 1995; Ranucci-Weiss et al., 1998]. Perinataly-acquired systemic adenovirus infections in neonates have also been described [Abbondanzo et al., 1989; Matsuoka et al., 1990; Abzug et al., 1991; Pinto et al., 1992; Rieger-Fackeldey et al., 2000]. Adenovirus has also been detected in tracheal aspirates from preterm neonates, within the first week of life, thus suggesting a possible mother to neonate vertical transmission [Couroukli et al., 2000; Prosch et al., 2002].

Several adenovirus serotypes are known to exhibit tropism for the genital tract, causing cervical infection in women and urethritis in men [Laverty et al., 1977; Swenson et al., 1995; Arnebrg et al., 1997; Bradshaw et al., 2002; Bradshaw et al., 2006]. Finally the expression of coxsackie–adenovirus receptor (CAR) in the villous trophoblast cells, which is a prerequisite for adenoviral infection of the placenta, has been demonstrated recently [Koi et al., 2001a; Koi et al., 2001b].

In spite of all these observations a definitive association between adenovirus placental infection and an adverse reproductive outcome has not been established. The purpose of this study was to investigate in a case-control study, the detection of adenovirus in placentas in conjunction with preterm birth and histological evidence of inflammation.

MATERIALS AND METHODS

Study design

During a twelve month period (1 January 2005 – 31 December 2005) all placental specimens from spontaneous preterm deliveries were collected prospectively and consecutively, at the time of delivery at the Alexandra University Hospital, Athens, Greece. Inclusion criteria consisted of women who carried singleton pregnancies and delivered with a preterm birth secondary to premature rupture of membranes, premature labour, or cervical insufficiency. Pregnancies with congenital abnormalities of the fetus were excluded. Preterm cases were divided in three subgroups according to gestational age (subgroup A: early preterm ≤ 29wks; subgroup B: intermediate preterm=30-33 wks; subgroup C: late preterm=34-36 wks). Gestational age was calculated by dates and by an ultrasound scan. For each preterm case, one or two full term specimens (≥ 37 weeks gestational age) were also collected consecutively at the same approximate day with preterm placentas (< 37 wks gestational age). For both groups various demographic characteristics, including maternal age, race, obstetric history, estimated gestational

age at delivery and fetal sex, were collected by the primary investigator (ET). The study obtained ethics committee approval and informed consent by all patients.

Placenta samples

A total of 193 placenta samples, including part of the chorionic plate and chorionic villi, were stored at -80°C for adenovirus genome detection. Before DNA extraction placenta samples were washed in order to remove maternal blood from the intervillous space. In 58 cases, the peripheral membranes were also sampled, then fixed in a 10% buffer formalin dilution and embedded in paraffin for pathological examination.

Placenta DNA extraction and polymerase chain reaction (PCR)

PCR was used for the detection of adenovirus genome in the placenta tissue. Placental DNA extraction and PCR analysis were blinded to the gestational age of the sample. DNA extraction on both case and control tissues was carried out using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA) according to the protocol provided by the manufacturer. In order to verify that adequate amounts of DNA were extracted from all samples, a control PCR amplification of the beta-actin gene was performed on all specimens. Adenovirus genome detection were performed using primers (hexAA1885: 5'-GCC GCA GTG GTC TTA CAT GCA CAT C-3'; hexAA1913: 5'-CAG CAC GCC GCG GAT GTC AAA GT-3') and cycling parameters as describe previously [Allard et al., 1990]. With this method DNA of almost all human adenovirus types derived from a wide range of clinical samples can be detected. All specimens were tested both neat and diluted by 10^{-1} in order to verify the absence of inhibition. Positive and negative controls were included in all PCR experiments and genetic material was extracted twice from each sample. In order to confirm the specificity of PCR products, the expected 308-bp adenovirus DNA amplicons were purified by a Qiaex gel extraction kit (Qiagen, Chatsworth, California, USA) and used as templates for sequencing of both strands with the ABI PRISM 377 DNA sequence analyzer (Perkin Elmer, Applied Biosystems Division, Foster City, California, USA).

Pathological examination

Thirty-seven preterm birth and 21 control placentas which contained part of the peripheral membranes were collected for histological examination of chorioamnionitis. Samples from the peripheral membranes stained with routine hematoxylin-eosin were blinded and evaluated by light microscopy to diagnose histological chorioamnionitis. The presence of neutrophils in the membranes (chorion and/or amnion) was used to define chorioamnionitis.

Statistical analysis

Variables entering the study were gestational age (preterm versus full term placentas), maternal age, the presence of adenovirus genome (PCR positive versus PCR negative), seasonal distribution and the presence of chorioamnionitis (positive versus negative). Differences between subgroups were evaluated using chi-square test. A P value < 0.05 was considered as statistically significant. Odds ratios (OR) and their respective 95% confidence intervals (CI) were calculated in order to estimate the magnitude of association between adenovirus genome detection and gestational age. Logistic regression was used to adjust for possible confounders, such as maternal age, in the detection of adenovirus. A statistical analysis was carried out using the statistical package STATA 8.0.

RESULTS

Characteristics of samples

During the study period 233 placenta samples were collected prospectively (98 preterm and 135 full term samples). All spontaneous preterm deliveries were included. From preterm placenta samples 48 were classified as late preterm (subgroup A), 30 as intermediate preterm (subgroup B) and 24 as very preterm (subgroup C). Of those, 28 late preterm, 3 intermediate preterm and 13 full term samples were excluded from the study because the inclusion criteria were not fulfilled, or due to inadequate sampling or storage procedure. Thus, 193 placenta samples (71 preterm and 122 full term placentas) which met the criteria were evaluated. From preterm placenta samples 20

were late preterm (subgroup A), 27 intermediate preterm (subgroup B) and 24 very preterm (subgroup C).

Mean maternal age at delivery was 29.7 ± 5.6 years. Mean maternal age at preterm deliveries was 29.2± 5.4 years, while in the full term controls was 30.8 ± 5.8 years (P >0.05). The two groups had similar demographic features and between preterm and full term cases there were no statistically significant differences as far as maternal age at birth, previous fetal loss or therapeutic abortions, previous gestations, nationality of mothers and fetal sex (P >0.05).

Molecular assays

Adenovirus genome was detected in 54 (28.0%) of the total samples. From the 71 placenta tissues of preterm birth, 29 (40.8%) were found positive for adenovirus genome, whereas from the 122 placenta tissues of full term controls only 25 (20.5%) samples were positive for adenovirus by PCR (P=0.002; Table 1). The probability of detecting adenovirus genome in preterm cases was estimated to be 2.7 times higher compared to term controls (OR=2.7, 95% CI, 1.4-5.1). A similar association appeared when the subgroups of very and intermediate preterm placentas were compared to full term controls (P=0.026; OR =2.8; 95% CI, 1.1-7.0 and P=0.026; OR =2.7; 95% CI, 1.1-6.5, respectively; Table 1). In the subgroup of the late preterm however, this association was no longer significant statistically (P=0.056; OR=2.6, 95% CI= 0.9-7.0; Table 1). It is also noteworthy that adenovirus detection was equally distributed among the subgroups of preterm placentas (Table 1).

Sequencing results of all adenovirus PCR-positive cases showed that the amplified sequences corresponded to the expected adenovirus hexon genome. Comparative genetic variability revealed that the amplified hexon gene sequences had similarities ranging from 97.7 to 100%. This variability between sequences almost excluded contamination events and confirmed validity of the study. Alignments of the amplified adenoviral sequences have also shown that most of the sequences corresponded to human adenovirus types of species C (n=41), while smaller numbers of them corresponded to human adenovirus of species B (n=8) and species A (n=5). The

distribution of the adenovirus species did not differ between cases and controls. Adenoviruses that belonged to other species were not amplified.

Multivariate logistic regression used to adjust gestational age for maternal age, showed that maternal age of full term or preterm cases did not affect significantly the detection of adenovirus genome (*P*=0.38). Adenovirus infection, among both preterm birth cases and controls, was more frequently detected during the spring months (March to June) following the seasonal distribution of adenovirus respiratory infections (Figure 1).

Histopathology

Table 2 presents results of histological choriomnionitis among 37 preterm and 21 term placenta samples. A statistically significant difference in the presence of chorioamnionitis was found between preterm and full term placentas (49% vs 19%; *P*=0.025). Within the group of preterm placentas, histological chorioamnionitis was significantly more common among adenovirus PCR-positive samples than among adenovirus-negative samples (75% vs 36%; *P*=0.026). Also, within the total adenovirus PCR-positive placentas, chorioamnionitis was more frequent in preterm compared to full term samples (75% vs 19%; *P*=0.003). However, among adenovirus PCR-negative placentas, the pathological findings did not differ significantly between preterm and full term samples (36% vs 20%; *P*=0.488).

DISCUSSION

In the present case-control matched study, the detection rate of adenovirus genome in tissues from preterm placentas was compared with healthy full term cases. Although previous studies indicated that adenovirus is related to adverse pregnancy outcomes [Van den Veyer et al., 1998}, this is the first prospective cohort study that documents an association between the presence of adenovirus genome in the placenta and spontaneous preterm birth. Baschat et al. [2003] reported an association between adenovirus isolation from the amniotic fluid and the presence of fetal echogenic liver lesions or neural tube defects, suggesting that adenovirus can be a fetal pathogen

not recognized previously. Severe adenovirus infection in preterm neonates has been documented in several case reports. Abbondanzo et al. [1989] described fatal adenovirus pneumonia in a preterm neonate born at 25 weeks gestation. In this study the autopsy findings revealed that alveolar and bronchiolar lining cells contain frequently intranuclear inclusions and an overwhelming adenovirus infection was confirmed by *in situ* DNA hybridization. There is also evidence that viral acquisition from the mother, perhaps via the genital tract, is an important mode of adenovirus transmission [Abzug et al., 1991]. Molecular studies revealed that adenovirus serotypes 19 and 37 have a tropism for the genital tract [Arnerg et al., 1997]. In addition adenovirus is recognised as a rare cause of cervical infection or urethritis in men [Swenson et al., 1995; Laverty et al., 1977; Bradshaw et al., 2002; Bradshaw et al., 2006]. Couroucli et al. [2000] isolated adenovirus from the tracheal aspirates of preterm neonates within the first week of life suggesting a possible vertical transmission from the mother to the neonate. Despite all the previous evidence, the role of adenovirus in preterm birth has not been investigated substantially.

In the present study, preterm placentas were almost three times more likely to host adenovirus genome. Stratification by gestational age to early, intermediate and late preterm revealed that the earlier the gestational age the stronger the extent of this association, whereas in late preterm cases the association between adenovirus detection and preterm birth was no longer significant. These results are in line with the consensus that the role of infection in the etiopathogenesis of preterm labor pertains to the lower gestational age [Goldenberg et al., 2003; Iams, 2003]. However, it should be pointed out that with the primer sequences that were used some exotic types of adenovirus might have been missed from either cases or controls. It is of note that the PCR protocol in the present study does not use degenerated primers that are able to amplify all adenovirus genotypes [Allard et al., 2001]. It should be also mentioned that some of our full term and preterm samples were excluded from the study mainly due to inadequate sampling or storage procedure. Since the exclusion of these samples was random and not related with the study group we believe that the elimination of these specimens did not influence our results.

The histological findings of the present survey showed that chorioamnionitis is associated with premature birth, as expected. This association remained significant in the subgroup of adenovirus PCR-positive samples, whereas it was no longer significant in the subgroup of adenovirus PCR-negative placentas. These findings reveal a meaningful association between adenovirus infection and histological chorioamnionitis, suggesting an etiological relation. Seropositivity data which would confirm this relation were not available in the present study. In a recent report [Arechavaleta-Velasco et al., 2008] adenovirus IgM antibodies were found more frequently in the serum of 24 women, who delivered preterm and sampled before 20 weeks of gestation. However, this study group was rather small and the sampling period was restricted for any safe conclusions to be drawn regarding the possible etiological role of adenovirus in preterm birth.

The finding of increased adenovirus detection between March and June, following the seasonal variation in respiratory adenovirus infections, supports previous similar observations in samples from amniotic fluid and tracheal aspirates of preterm neonates [Prosch et al., 2002; Baschat et al., 2003a]. In conclusion, this study documents an association between adenovirus insult and preterm labour, possibly by causing chorioamnionitis expanding the spectrum of infectious pathogens which are related traditionally to chorioamnionitis. Further studies are warranted to investigate the pathogenic mechanisms relating adenovirus infection during pregnancy with placental inflammation and premature birth.

No conflict of interest for all authors.

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Table 1. Adenovirus genome detection in all preterm and in preterm subgroups according to gestational age vs full term controls.

Gestational age (wks)	PCR-positive *n* (%)	PCR-negative *n* (%)	Total	*P*	Odds ratio	95% CI
Term ≥ 37	25 (20.5 %)	97 (79.5 %)	122			
Preterm < 37	29 (40.8 %)	42 (59.2 %)	71	**0.002**	2.7	1.4-5.1
Subgroup A: 34-36	8 (40.0%)	12 (60.0%)	20	0.056	2.6	0.9-7
Subgroup B: 30-33	11 (40.7%)	16 (59.3%)	27	**0.026**	2.7	1.1-6.5
Subgroup C: 29-22	10 (41.7%)	14 (58.3%)	24	**0.026**	2.8	1.1-7

Table 2. Occurrence of histological chorioamnionitis in placenta tissues among different study groups.

	Histological choriamnionitis			
	Positive *n* (%)	Negative *n* (%)	Total *n*	x^2 (*P* value)
Overall sample (*n*=58)				
Preterm	18 (49%)	19 (51%)	37 (64%)	
Full term	4 (19%)	17 (81%)	21 (36%)	4.99 (**0.025**)
Adenovirus PCR-positive group (*n*=28)				
Preterm	9 (75%)	3 (25%)	12 (43%)	
Full term	3 (19%)	13 (81%)	16 (57%)	8.86 (**0.003**)
Adenovirus PCR-negative group (*n*=30)				
Preterm	9 (36%)	16 (64%)	25 (83%)	
Full term	1 (20%)	4 (80%)	5 (17%)	0.48 (0.488)
Preterm group (*n*=37)				
Adenovirus PCR-positive	9 (75%)	3 (25%)	12 (32%)	
Adenovirus PCR-negative	9 (36%)	16 (64%)	25 (68%)	4.94 (**0.026**)

FIGURE LEGENDS

Fig 1. Bar graph depicting the seasonal distribution of adenovirus genome detection in preterm and term placentas.