Type I Diabetic Pregnancy and Subclinical Human Papillomavirus Infection

Sakari Hietanen, Ulla Ekblad, Tarja-Terttu Pelliniemi, Kari Syrjänen, Hans Helenius, and Stina Syrjänen

It has been suggested that diabetic pregnancy is an immunosuppressive state. To determine whether the possible immunosuppression in pregnant diabetics might result in an increased risk for human papillomavirus (HPV) infection, we studied exfoliated cells from the uterine cervix, vagina, and posterior commissure of the vulva by means of dot blot hybridization with use of a probe cocktail of HPV types 11, 16, and 18 under low stringency and by means of consensus primer-mediated polymerase chain reaction (PCR) targeted to the HPV L1 and E1 regions. For this study, samples from 31 pregnant diabetics whose glucose levels had been reasonably well controlled were analyzed during the first trimester; samples from 27 of these patients were analyzed again during the third trimester. Fifty-one healthy pregnant women were included as controls. Only one of the pregnant diabetics was positive for HPV DNA. The L1 PCR products of the first and third trimester samples from this patient were sequenced, and both were found to represent HPV 61. Three of the pregnant controls were positive for HPV: two were positive for HPV 16, and one was positive for HPV 6. The 95% confidence limits for the prevalence of HPV were calculated to be 0.1%–16.7% for the diabetics and 1.2%–16.2% for the controls. The 95% confidence limits for the difference between the groups were −11.6%–6.3%. These results suggest that pregnant diabetics do not have an increased risk of developing HPV infection, at least when their glucose levels remain well controlled.

In pregnant diabetics, abnormalities in cell-mediated immunity as well as the immunologic modifications associated with normal pregnancy concurrently affect immunologic status [1]. Alterations of immunoregulatory T cell subsets have been detected in peripheral blood samples from patients with newly diagnosed diabetes and from diabetic pregnant women [2–4]. A recent study showed alterations in cell-mediated immunity in pregnant diabetics, as indicated by T cell subset determinations and lymphokine production after phytohemagglutinin stimulation [5].

If diminished immune responsiveness does depress resistance in diabetic mothers, more frequent infections would result. Infections may also disseminate more easily, and latent viral infections may reactivate more readily. In addition, infections with human papillomavirus (HPV) might be more frequent in pregnant diabetics than in healthy pregnant women. Prevalence studies of HPV in pregnant women have produced contradictory results, showing either an increase in the frequency of HPV infections [6, 7] or no change [8, 9]. Despite many reports on the prevalence of HPV infection among pregnant women, to our knowledge, no studies exist on the prevalence of HPV infection among pregnant diabetics. Increased prevalence and severity of HPV infection have been reported for immunosuppressed patients [10, 11]. If diabetic pregnancy is associated with clinically significant immunosuppression, an increase in the occurrence of HPV infection would be expected. Therefore, we conducted a study to determine the frequency with which HPV DNA was present in pregnant diabetics during the first and third trimesters to evaluate whether the presence of diabetes in pregnant women results in a higher frequency of HPV infection.

Patients and Methods

This study was conducted at the Turku University Central Hospital (Turku, Finland). The samples for HPV DNA analysis were collected during the period October 1991—March 1993. All pregnant diabetics in the catchment area of this hospital (~900,000) were included in the study. In Finland, follow-up of diabetic pregnancies is conducted at the university clinics. The study group consisted of 31 Caucasian, middle-class pregnant women with insulin-dependent diabetes who were referred to the Central Hospital for antenatal care. Diabetic pregnancy was classified according to the method of White [12], which is used to predict pregnancy complications in diabetics. All of the patients were married or were in common-law marriages. Twenty of the diabetics were primiparous. One

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Informed consent was obtained from the patients, and the design of this study was approved by the Hospital Ethics Committee of the Turku University Central Hospital, Turku, Finland.

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precipitated by adding 300 μL of saturated NaCl (0.4M). After centrifugation, DNA was precipitated from the supernatant with 30% ethanol. and proteinase K (0.3 mg/mL) overnight at 37°C. Proteins were stored at -20°C until processed. Samples were lysed in 1 mL (0.02 mg/mL), vortexed, and centrifuged. The cell pellet was centrifuged for 4.5 minutes at 95°C and then exposed to 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 50 seconds, and extension at 72°C for 60 seconds in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). The PCR cycle for amplification. The reaction took place on 300 ng of DNA in a 50-μL reaction volume. The PCR solution contained 5 μL of 10 X PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH, 8.3], 1.5 mM MgCl₂, and 0.1% Triton X-100), 0.8 units of DynaZyme DNA polymerase (Finnzymes, Espoo, Finland), 200 μM each deoxynucleotide triphosphate, 20 pmol of the primers and sterile water.

The template DNA for MY09, MY11 PCR was first denatured for 4.5 minutes at 95°C and then exposed to 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 50 seconds, and extension at 72°C for 60 seconds in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). The PCR cycle for p1E1, p2E1 primers consisted of initial template DNA denaturation for 3.0 minutes at 94°C and then 34 cycles of denaturation at 94°C for 1 minute; 2 minutes of annealing at 42°C; and extension for 1.5 minutes at 72°C. The amplifications with both primer sets were completed by a 7-minute extension step at 72°C. Target DNAs for the positive controls were HPV plasmid

Table 1. Clinical data on pregnant diabetics who were screened for human papillomavirus infection.

<table>
<thead>
<tr>
<th>Variable</th>
<th>First trimester (n = 31)</th>
<th>Third trimester (n = 27)</th>
<th>Controls (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (y) ± SD*</td>
<td>27.6 ± 4.0</td>
<td>28.1 ± 4.1</td>
<td>28.3 ± 4.6</td>
</tr>
<tr>
<td>Mean duration of gestation (w) ± SD</td>
<td>8.3 ± 2.4</td>
<td>8.8 ± 1.9</td>
<td>8.4 ± 9.0</td>
</tr>
<tr>
<td>Median duration of gestation (w)</td>
<td>7</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>No. with indicated diabetes classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class B</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Class C</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Class D</td>
<td>16</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Mean hemoglobin A1c values ± SD</td>
<td>6.9% ± 1.3%</td>
<td>6.2% ± 1.0%</td>
<td></td>
</tr>
</tbody>
</table>

* The differences in age of first trimester pregnant diabetics and controls were not significant (P = .48).
DNA and DNA extracted from CaSkI cells (HPV 16-positive cell line). No DNA was added to the negative controls. Both the undigested and digested PCR products were electrophoresed in 3% agarose gel (NuSieve, FMC BioProducts, Rockland, ME). Bands were visualized by ethidium bromide staining.

The specificity of the PCR products was confirmed by restriction fragment mapping with use of the PstI and Rsal enzymes for cleavage. The DNAs of positive PCR products were transferred to a filter and hybridized using a mixture of whole genomic biotinylated HPV 6/16/18 probe under low stringency conditions (T_m = −35°C). The hybrids were visualized with streptavidin-alkaline phosphatase complex by using nitroblue tetrazolium as the chromogen and 5-bromo-4-chloro-3-indolyl phosphate as the substrate [15]. The sensitivity of the PCR was tested with MY09, MY11 primers and CaSkI cells. We mixed CaSkI cells (10,000, 1,000, 100, 50, 10, 5, and 1) with 300 ng of DNA extracted from healthy human fibroblasts and performed PCR as described above. PCR was still positive when one CaSkI cell was used. In addition, DNA extracted from 20 SiHa cells (1–2 copies of HPV 16 per cell) resulted in a visible amplification band in the gel (data not shown).

Direct DNA sequencing was performed with the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical, Cleveland, OH) according to the manufacturer’s instructions. PCR amplification was performed with MY09, MY11 primers. DNA fragments were resolved by polyacrylamide gel electrophoresis and exposures to X-ray films were made at −70°C.

Confidence intervals for HPV prevalence were calculated with use of Fisher’s exact test and binomial distribution.

**Results**

On clinical examination, none of the diabetic or control mothers had visible warts. A first-trimester Pap smear showed mild atypia (atypical squamous cells of undetermined significance [ASCUS], according to the Bethesda system) for only one of the 31 pregnant diabetics. A later Pap smear, performed near term for this patient, showed no atypia. Pap smears were performed for 30 healthy pregnant controls, and four of these smears showed ASCUS cytology. HPV DNA could be detected in only one sample (obtained from a control) in which ASCUS was present. None of the investigated Pap smears showed classic signs of HPV infection, and no higher grades of atypia were detected either among the diabetics or among the controls. We also tested all patients for chlamydia antigen and found no positive samples.

Dot blot hybridization was performed under low stringency for all samples with use of the HPV 11, 16, and 18 probe mixture. Two samples from the group of pregnant diabetics were positive. These two samples were obtained from the same patient during the first and third trimesters of pregnancy. One sample in the control group was positive by dot blot hybridization.

PCR successfully amplified the β-globin gene in all scrapings. Of the 121 samples, 10 could be amplified with the MY09, MY11 consensus primers for HPV DNA. Only five of these positive samples could be amplified with HPV consensus primers targeting the E1 region. Electrophoretic analysis of the MY09-MY11 amplimers revealed the expected size of the PCR products, but restriction mapping with the PstI and Rsal enzymes matched HPV for only three samples. These samples were obtained from controls; HPV 6 was present in one of the samples, and HPV 16 was present in two. Only these three PCR products hybridized to the HPV 11, 16, and 18 probe mixture under low stringency.

The two samples whose restriction fragments did not match HPV were derived from the same patient. They were positive both by dot blot hybridization and by PCR and were further analyzed by direct sequencing of the MY09-MY11 PCR products. The sequence was found to be closely homologous to HPV 61.

The prevalence of HPV was 3.2% among pregnant diabetics and 5.9% among healthy pregnant controls. The 95% confidence limits for the prevalence of HPV were 0.1%–16.7% and 16.7% for diabetics and 1.2%–16.2% for controls. The 95% confidence limits for the difference were −11.6% to 6.3%.

**Discussion**

In this study we found a low frequency of HPV infection, and we failed to detect any increase in the frequency of the infection during advanced gestation in pregnant diabetics. Moreover, we did not detect more HPV infections in this group than in healthy pregnant controls. In previous studies the prevalence of HPV infection during pregnancy in healthy women has varied between 10% and 28% [6–9, 16, 17]. We found that the frequency of HPV infection was 6.0% among the pregnant controls and 3.3% among the pregnant diabetics. Melkert et al. [18], who also used PCR to study HPV infection, have reported a prevalence of the infection that ranges from 4.1% to 6.6% among women aged 15–34 years who have healthy cervices.

Our results parallel this finding, although it must be emphasized that in our study the Pap smear was merely a method to search for typical cytological changes associated with HPV infection, not an exclusion criterion. Nevertheless, none of the investigated Pap smears from our patients showed signs of HPV infection. The low frequency of HPV infection in both diabetic mothers and in controls may be explained partly by the fact that the groups consisted of women without known sexual risk factors, as evidenced by the absence of previous STDs and the fact that all mothers were married or were in common-law marriages.

In Finland in 1992, 60% of women aged 25 to 34 were married, and the number of sexual partners during a lifetime was 1–5 for 70% of women; 70% of women had had only one sexual partner during the past year [19]. Moreover, pregnant
diabetics usually are in monogamous relationships because their pregnancies should be planned to avoid diabetic embryopathy.

We detected three PCR-positive samples in the diabetes group and two in the control group with use of the MY09, MY11 primers, but the PCR products or the original sample DNA did not hybridize to HPV screening probes under low stringency. Digestion of the PCR products produced restriction fragments that did not match any known HPV types. In addition, PCR targeting the E1 region gave a negative result. Therefore, the amplification product was of non-HPV origin. Without the thorough analysis of the positive samples, the rate of false positives would have increased the frequency of HPV in both groups significantly.

It may be that when the possible nonspecific PCR amplifications observed in other studies are taken into account, the true prevalence of HPV among pregnant women with cytomorphologically normal cervices is in fact below 10%, at least in some geographic regions among women who are married or are in common-law marriages and have no histories of STDs.

In our study, the frequency of HPV among diabetics and controls was quite similar. Estimating the prevalence of HPV is difficult with a small number of patients; however, on the basis of the 95% confidence limit, we can say that the prevalence is between 1% and 16% for both diabetic and nondiabetic pregnant women in Finland. Given the rate of HPV infection among the controls (i.e., 3 [6%] of 51), the rate of HPV infection among the diabetic mothers would have to have been at least 7 (23%) of 31 to result in a statistically significant increase ($P < .05$) in the prevalence of HPV infection (95% confidence limits, 0.6%–32.8%).

The results of this pilot study therefore suggest that diabetic pregnancy is not associated with a clinically significant increase—if any increase—in the prevalence of HPV over that observed among healthy pregnant women, at least when the diabetics are under control. Because this study included a relatively small group of patients, the results that were obtained should be verified in other studies with larger numbers of patients.

References