CHAPTER VII

ROLE OF RETINOIC ACID IN HPV-MEDIATED CERVICAL CANCER

INTRODUCTION

Although HPV infection has been established as a central risk factor of cervical cancer, there is much interest in the role of diet in its etiology. The persistent HPV infection appears to confer an elevated risk, and cofactors at the nutritional level may be necessary to allow the virus infection to progress to cervical cancer (Giuliano et al., 1997). Both experimental, as well as in vivo studies, have suggested that the lower dietary intake of Vit. A may increase the risk of cervical cancer (Hong & Itri, 1994, Moon et al., 1994; Creeket et al., 1995, Lehtinen et al., 1999). A protective role of vitamin A in the subsequent development of various type of cancers, particularly those of epithelial origin including cervix has been suggested from several lines of evidence (Shimizu et al., 1996, Nagata et al., 1999).

An inverse association was reported for the serum retinol level with risk of cervical cancer (Shimizu et al., 1996) and its progression (Kwasneiwiska and Tukendorf 1996; Ramaswamy and Krishnamoorthy 1996). It was also suggested that the HPV may be an effective modifier of risk for cervical cancer (Kwasniewska and Tukendorf 1996; Lehtinen et al., 1999; Nagata et al., 1999). A reduction in cell proliferation has also
been observed in vit. A (retinol) treated HPV immortalized epithelial cell lines (Mason et al., 1999). The dietary form of vitamin A (retinol, retinyl palmitate) must be metabolized to retinoids, which are the actual ligands for the nuclear receptors (Sporn and Robert, 1994).

Retinoids are active metabolites of vitamin A (retinol), which includes all-trans-retinoic acid (ATRA), 9-cis-retinoic acid, 13-cis-retinoic acid etc, capable of profound impact on many biological functions like anti-proliferative, differentiative and immuno modulatory properties (Ahn et al., 1997). Naturally occurring retinoids regulate the growth and differentiation of a wide variety of cell types and play a crucial role as morphogenic agents during embryonic development (Lee et al., 1995; Evans and Kaye, 1999). Retinoids exert most of their effect by binding to specific receptors and modulating gene expression (Evans and Kaye, 1999). These retinoid receptors namely RAR-α, β, γ and RXR α, β, γ (Chambon, 1995) are nuclear transcription factors, mediate the ability of retinoids to control cellular proliferation and differentiation by interacting with retinoid response elements of gene (Mangelsdorf et al., 1994). At the cellular level, activation of these retinoid receptor can inhibit cell proliferation, induce differentiation and apoptosis in epithelial cells during normal cell development as well as in transformed cells in tissue culture (Evans and Kaye, 1999). Retinoids are also able to induce p21, an effector of p53 and consequently, cause growth arrest and differentiation (Shao et al., 1995, Liu et al., 1996) However, the mechanism of induction of apoptosis remains unclear.
Both natural retinoic acids and their synthetic analogues are being investigated as chemotherapeutic agents (Dawson et al., 2001; Nagpal and Chandraratna, 2001) as well as chemopreventive drugs (Narayanan et al., 1998) because of their anti-proliferative and apoptosis inducible activity. Particularly ATRA has a potent role as a cell cycle regulator and an anti-viral chemopreventive agent in a wide variety of cell types (Narayanan et al., 1998; Hofmanova et al., 2000). A cell line study has also demonstrated that the retinoic acid treatment is capable of improving stress fiber formation, decreasing cell detachment and increasing cell adhesion capability (Matarrese et al., 1998).

The mechanism of retinoid function in HPV-associated cervical cancer is however unclear. In order to understand the role of retinoic acid in cervical cancer it is necessary to analyse its status in HPV associated cervical cancer and its progression in comparison with normal level. The present study was therefore focused on the analysis of vitamin A metabolite ATRA in serum of HPV positive and negative cervical lesions and in normal subjects.

WORKING HYPOTHESIS

The potential role of nutrition as an additional independent risk factor for cervical cancer has not been appropriately addressed in the context of HPV infection. Since cervical cancer remains a problem among lower socio-economic women (Brinton and Hoover, 1992), the nutritional status plays a major role in it. Studies on the effect of dietary intake of Vit.A on the risk of cervical cancer or dysplasia, have yielded conflicting
results. Some have reported that the lower intake of dietary retinol or β-carotene was associated with an elevated risk of cervical cancer (Marshall et al., 1983; Lu et al., 1993; Hong and Itri, 1994; Moon et al., 1994; Creek et al., 1995; Shimizu et al., 1996; Ramaswamy and Krishnamoorthy, 1996; Lehtinen et al., 1999). However, vitamin A exerts its effect on cell growth control only through its metabolite called retinoids, \textit{in vivo}. A good relation has been shown between these retinoids especially ATRA and HPV infection in many \textit{in vitro} cell culture studies (Creek et al., 1995; Narayanan et al., 1998). It has been suggested that retinoid inhibit cervical cancer cell growth through repression of AP-1 transcription factor activity, mediator of HPV infection or by down regulation of EGFR expression. It is also believed that ATRA can inhibit immortalization of human epidermal keratinocytes during or after transfection with HPV 16 (Creek et al., 1994) and can act as an anti-promoter by inhibiting progression of papilloma to carcinoma (DeLuca et al., 1994).

However, in cervical cancer, the HPV infection somehow overcomes the feedback anti-viral activity (Hofmanova et al., 2000) of retinoids and causes cervical cancer. This modulatory capacity of HPV may vary among the subtypes with respect to its oncogenic potency. The present study was therefore carried out to analyse the serum level of ATRA (metabolite of vit. A) in HPV-associated cervical cancer.
STUDY DESIGN

The present study was designed to assay the level of ATRA in the serum of the total study population of 16, including normal age matched control using HPLC technique. The study population was grouped as follows.

- **Group I**: Normal age matched control (4)
- **Group II**: Severe dysplasia without HPV infection (4)
- **Group III**: Cervical cancer with HPV 16/11 infection (4)
- **Group IV**: Cervical cancer with HPV 16/18 infection (4)

The HPV status of the study population was analysed already (Chapter III) by PCR technique.

Sample Collection

5 ml of blood was collected by venepuncture from the patients whose biopsy had been taken for HPV analysis from the gynecologic OP and transferred to the lab by keeping in ice. Serum was isolated immediately by centrifugation and analysed freshly or stored at -70°C until use (2-3 days).

METHODOLOGY

Serum retinoic acid (ATRA) was analysed using reversed phase high performance liquid chromatography (HPLC) based on the method of Frolik et al. (1978) and Nells and Leenheer, (1983). The wavelength of
313 nm was selected for the detection of ATRA, based on the retinoid separation by HPLC (McCormick et al., 1980). The sample preparation for HPLC analysis was done according to the standard protocol for vit. A extraction (McCromick et al., 1980 and Miller Yang 1985). The detailed protocol is given in Appendix. The amount of ATRA in the sample was calculated from the absorbance peak ratios of standard and sample.

RESULTS

In the present study, we have observed a prominent absorption peak at a retention time (RT) 12.5 for standard ATRA and the area of the peak lies in the range of 11 to 13, which is very much consistent with the standard methods. In all the four normal samples of Group I analysed, we got the peak at the RT range 11-12.5. From this we have concluded that the peak at RT around 12 is specific for ATRA. A representative run of the normal serum HPLC shows multiple peaks at RT 4.5, 7.5, 11.2 & 14 and is given in (Fig.19b) where, the 11.2 RT specific for ATRA was also detected. Similarly all the cancer samples (Group II, III & IV) analysed also showed an absorption peak at 11-13.5 RT range (Fig.19c,d,e). Very little reduction in the area of peak was seen in Group II when compared to Group I. But in Group II the area of peak was much lower than that of normal sample (Group I) and an even more reduced peak area was observed in Group IV (Fig.19e). The concentration of ATRA was calculated in all these groups from the peak area and concentration of standard ATRA and summarised in Table 17.
a. Standard all-trans-retinoic Acid peak at a range of 11-14 RT.

b. Group - I Serum of normal subjects shows an absorbance peak at a range of 11-13 RT.
c. Group - II  Serum of cervical cancer patients without HPV infection shows an absorbance peak at a range of 12-14 RT.

definition

d. Group - III  Serum of cervical cancer patients with HPV 6/11 infection shows an absorbance peak at a range of 12-14 RT.

definition

e. Group - IV  Serum of cervical cancer patients with HPV 16/18 infection shows an absorbance peak at a range of 12-14 RT.
Table 17: Levels of ATRA in the Serum of Normal and Different Groups of Cervical Cancer Sera

<table>
<thead>
<tr>
<th>Study Subject</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.659</td>
<td>0.610</td>
<td>0.309</td>
<td>0.273</td>
</tr>
<tr>
<td>2</td>
<td>0.632</td>
<td>0.603</td>
<td>0.296</td>
<td>0.256</td>
</tr>
<tr>
<td>3</td>
<td>0.613</td>
<td>0.612</td>
<td>0.301</td>
<td>0.242</td>
</tr>
<tr>
<td>4</td>
<td>0.641</td>
<td>0.594</td>
<td>0.184</td>
<td>0.261</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.636 ± 0.017</td>
<td>0.605 ± 0.007</td>
<td>0.298 ± 0.009</td>
<td>0.258 ± 0.011</td>
</tr>
</tbody>
</table>
DISCUSSION

In the present study, we have analysed the serum level of ATRA in normal subjects and in cervical lesions of different groups. Table 17 shows the serum levels (µg/ml) of individual cases and the mean value of each group studied. From the results it is seen that the serum ATRA level of group II patients, without HPV infection was more or less similar (0.605 ±0.007) to that of normal subjects (group I). Whereas, in group III cases, infected with low-risk HPV 6/11, the serum levels of ATRA were found to be decreased when compared to Group II with a mean value of 0.298 ± 0.009. Interestingly, a drastic reduction in serum ATRA level has been indicated with a mean value of 0.258 ± 0.011 when compared to other groups. It is important to note that the 3 cases of group IV were with premalignant cervical cancer lesions. We could not find the statistical correlation between HPV infection and ATRA level, because of lesser number of study subjects. From this result, we suggested the depletion or reduction in serum ATRA may have a direct link with HPV infection and may depend on the oncogenic potency of HPV subtypes.

Earlier studies also have pointed out the association of retinoic acids with cervical cancer (Sporn and Roberts, 1994) and HPV infection (Narayanan et al., 1998). Both ATRA and 9-cis-retinoic acid were found to inhibit cell proliferation in cervical cancer cells by inducing cell cycle arrest, suggesting its direct effect on p53 function. (Narayanan et al., 1998). It was also suggested that the plasma level of retinol can modulate the progression of latent HPV infection to subclinical lesions and CIN (Kwasniewska et al., 1997). Meyskens et al. (1994) reported that the
Topical application of ATRA can enhance regression of dysplasia of the uterine cervix. There are several pathways whereby RA can modulate the expression or activity of HPV E6 and E7 transforming proteins (Howley, 1991). It is also shown that RA can directly downregulate HPV messenger RNA (Bartsch et al., 1992). Retinoids inhibit cervical cancer cell growth by downregulating EGFR (Creek et al., 1995).

HPV infection overcomes the anti-viral activity of RA to establish its effect in cervical cancer. This may be due to the lack of retinoic acid in serum which may be the reflection of lower dietary intake of its precursor vitamin A (retinol) or the interruption of metabolic pathway of retinoic acid by HPV infection. Inspite of intensive investigation on anti-proliferative activity of retinoic acid in vitro, the role of retinoic acid in HPV-induced cervical cancer is not yet identified. The results of the present study may pave the way for the understanding of the relationship between retinoic acid and HPV infection and needs further clarification. The ATRA can be targeted as an effective anticancer therapy (locally or systematically) in HPV-associated cervical cancer.