Analysis of Interleukin 6 Gene Expression in Cervical Neoplasia Using a Quantitative Polymerase Chain Reaction Assay: Evidence for Enhanced Interleukin 6 Gene Expression in Invasive Carcinoma


ABSTRACT

Interleukin 6 (IL-6) is a multifunctional cytokine which has recently been shown to act in vitro as a growth factor for cervical carcinoma cell lines. This prompted us to measure IL-6 gene expression using a new quantitative polymerase chain reaction assay in 13 invasive cervical cancers, 5 cases of cervical intraepithelial neoplasia, and 2 normal cervix. A significant increase in the expression of the IL-6 gene in invasive cervical carcinoma as compared to cervical intraepithelial neoplasia and normal cervix was demonstrated (P < 0.05). Unlike IL-6, the expression of other cytokine genes such as γ-interferon was not correlated with any particular cervical histological lesion. Immunohistochemical analysis identified IL-6 protein only on stroma cells which, based on morphological criteria, most likely belong to the macrophage lineage. This was reinforced by the correlation observed between IL-6 gene expression and macrophage tumor infiltration (P < 0.007). No IL-6 immunostaining of cervical tumor cells was shown. Therefore this study confirms, in vivo, that IL-6 may play a role in the pathogenesis of carcinoma of the uterine cervix since its increased expression is associated with advanced neoplastic cervical lesions. In contrast to in vitro studies, the stromal origin of IL-6 suggests that this cytokine may modulate tumor cell proliferation by a paracrine rather than an autocrine mechanism.

INTRODUCTION

Cervical cancer is the second most common female cancer, with 500,000 new cases per annum worldwide (1). Since 1986, its incidence in white women under the age of 50 years has reversed its previous downward trend and has been increasing (2). A factor that appears to play an important role in the development of this cancer is infection with certain types of HPV (3, 4). The DNA of HPV types 16, 18, and 33, and less frequently other types, can be found in about 85% of cases of cervical neoplasia. Nevertheless, most researchers agree that HPV infections are not sufficient for cancer induction, and other factors are necessary to induce CIN to progress to invasive cancer. Among potential cofactors, amplification of oncogenes such as c-myc and erbB-2 has been reported in carcinomas of the uterine cervix and is associated with poor prognosis (5–8). It is noteworthy that in invasive cervical carcinoma, HPV DNA is often found integrated near cellular oncogenes and may activate them (9).

Cytokines, such as IL-6, have also been involved in the modulation of tumor development. IL-6 is a multifunctional cytokine produced by various types of lymphoid and nonlymphoid cells including some tumor cell lines (10). It induces a variety of acute phase proteins such as C-reactive protein and fibrinogen and has been reported to potentiate the in vivo growth of cell lines derived from multiple myeloma, ovarian carcinoma and renal carcinoma (11–13). The presence of high serum levels of IL-6 is an adverse prognostic factor in multiple myeloma, renal cancer, melanoma, glioblastoma, ovarian cancer, and lymphoma (14–19). Various arguments suggest that IL-6 may also play a role in cervical carcinoma. Indeed, Hernandez et al. (20) showed that thrombocytosis could constitute an independent indicator of poor prognosis in patients with cervical cancer. Although the specific mechanisms by which thrombocytosis is produced in cancer patients are not definitively established, many studies indicate that IL-6 may be involved in some of these cases of paraneoplastic thrombocytosis (21). Moreover, elevated levels of serum IL-6 have been found in cervical cancers (18). Finally, it has been reported that IL-6 may function in vitro as an autocrine growth factor in cervical carcinomas (22).

Therefore, in order to more accurately define the role of IL-6 in the pathogenesis of cervical carcinomas, we addressed the question of IL-6 gene expression in normal cervix, CIN, and invasive cervical carcinoma. In situ hybridization techniques and immunocytochemistry are only semiquantitative and, since cytokine gene expression in tumors is often low, we developed a quantitative PCR assay for cytokine measurement based on the coamplification to saturation of an internal DNA standard highly homologous to the cDNA to be measured. This increases the sensitivity of detection of cytokine mRNA and allows accurate mRNA quantitation (23). We believe that this in situ IL-6 quantitation is complementary to serum IL-6 determination because natural inhibitors of cytokines present in sera, such as soluble cytokine receptors, can interfere with some cytokine assays (24, 25). Furthermore, cytokines should not be regarded as endocrine hormones because they often exert their function in the context of an intimate interaction between cytokine-producing and responding cells and they need therefore to be quantified at the tumor site (26).

Using this methodology, we report a significant increase in the expression of the IL-6 gene in invasive cervical carcinoma as compared to CIN and normal cervix, which did not seem to be related to secretion of IL-6 by tumor cells, but rather reflected the intensity of macrophage infiltration.

MATERIALS AND METHODS

Patients and Tissues. Cervical biopsy specimens were obtained from 18 patients. They included 13 patients with invasive squamous cell carcinoma and 5 patients with CIN, 2 of whom were CIN 2 and 3 were CIN 3, according to the International Federation of Gynecology and Obstetrics guidelines. Two tissue specimens of normal cervical epithelium were also analyzed. All invasive tumors were Stage I or II, i.e., limited to the cervix of partly extending to the vagina and/or parametrium.

Tissue specimens were immediately divided into two parts, one portion being fixed in formalin for histological analysis and immunocytochemistry and the other snap-frozen in liquid nitrogen and stored at −70°C for RNA extraction.
IL-6 GENE EXPRESSION IN CERVICAL NEOPLASIA

Immunocytochemistry. After deparaffinization and rehydration, the sections were progressively incubated at room temperature for 15 min with methanol containing 3% hydrogen peroxide to inhibit endogenous peroxidase. Staining was then performed using the universal rabbit/mouse-labeled streptavidin-biotin method (DAKO LSAB kit; DAKO Corp., Santa Barbara, CA). Between each step, sections were washed with phosphate-buffered saline solution. Finally, slides were counterstained with Harris hematoxylin, dehydrated, given a coverslip, and examined by conventional light microscopy.

Expression of IL-6 was determined by staining cells with polyclonal anti-IL-6 antibodies from Genzyme (Boston) as described previously (27). The specificity of the IL-6 staining reaction was confirmed by studies of the same sections with preimmune rabbit serum. In addition preabsorption of the IL-6 antiserum with recombinant IL-6 abolished specific staining.

We characterized the tumor immunological infiltrates using antibodies to T cells (anti-CD3 rabbit polyclonal antibody at a 1:100 dilution; Dakopatts) and macrophages (anti-CD68 monoclonal antibody, Kp1 at a 1:50 dilution; Dakopatts) in 19 cervical tissue sections. CD68-positive cells possessing a mononuclear cytomorphology were evaluated by counting random high-power fields with an American Optical microscope using a ×45 objective with a 0.47-mm diameter field. A cutoff value of 10 cells/field identified tumors with low or high T cell or macrophage infiltrates.

Preparation of RNA and cDNA Synthesis. Total cellular RNAs were extracted using the RNAzol B method based on the technique previously described by Chomczynski and Sacchi (28). Five μg of total cellular RNAs were reverse transcribed with random hexadeoxynucleotide primers using the reverse transcriptase (RT) kit from Strategic (1 μl Mg2+ in Promega buffer). PCR cycles were 1 min at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The reaction mixtures were overlaid with 50 μl mineral oil. The PCR cycles were reverse transcribed with random hexadeoxynucleotide primers using the reverse transcriptase (RT) kit from Strategic (1 μl Mg2+ in Promega buffer). The reaction was performed in a Perkin Elmer/Cetus DNA thermal cycler for 40 cycles and ended with a step of 10 mm at 72°C. Then, 2 μl of the amplified products would differ in size (Fig. 1). The specificity of the PCR was guaranteed by the size of the PCR product and confirmed by the use of an internal primer for the run-off reaction. The number of β actin transcripts was measured with the same methodology, and subsequently used to normalize IL-6 and IFNγ copy number. The limit of sensitivity of the quantitative PCR assay for IL-6 and IFNγ was 1 mRNA copy and 0.01 mRNA copy/106 β actin mRNA copies, respectively, in biopsy specimens.

A number of precautions were taken to avoid PCR artifacts. These included use of aerosol-resistant pipette tips, assembling reactions in laminar flow hoods, use of aliquoted reagents, and pipettes dedicated for PCR use. Negative controls consisting of buffer alone and/or nonreverse-transcribed sample RNA were included in each experiment.

HPV Typing. HPV typing was performed using Southern blot hybridization and PCR with primers specific for 6, 11, 16, 18, and 33 HPV types or consensus primers for HPV.

Statistical Analysis. Comparisons between IL-6 or IFNγ mRNA levels and different cervical lesions were assessed using the Mann-Whitney U test. Relationships between IL-6 gene expression, immune cell infiltration, and presence of HPV genome were analyzed using the χ² test with Yates correction when necessary. P < 0.05 was considered to be significant.

RESULTS

Comparison of IL-6 Gene Expression in Invasive Cervical Carcinoma, CIN, and Normal Cervix. By means of a quantitative PCR assay, an increased expression of IL-6 gene was found in invasive cervical carcinoma versus CIN or normal cervix. Indeed, only 1 of 7 patients in the CIN or normal cervix group had a detectable IL-6 gene expression, whereas 10 of 13 primary invasive cervical carcinomas expressed more than 1 IL-6 mRNA copies/106 (3 actin mRNA copies, respectively, in biopsy samples and for sample integrity control (29).

Expression of IL-6 gene was observed among invasive cervical carcinomas, as indicated by the Mann-Whitney U test. Relationships between IL-6 gene expression, immune cell infiltration, and presence of HPV genome were analyzed using the χ² test with Yates correction when necessary. P < 0.05 was considered to be significant.

Table 1 Oligonucleotides of 5' primers and 3' primers of four target genes

<table>
<thead>
<tr>
<th>mRNA species</th>
<th>Size of PCR product</th>
<th>Size of run-off product</th>
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<tbody>
<tr>
<td>IL-6 5'-ACGAAATCACAACACAAAAACACATGTCACA</td>
<td>335</td>
<td>125-129</td>
</tr>
<tr>
<td>IL-6 3'-CATCTAGAGTTTCGTCTTGTGCC</td>
<td>335</td>
<td>125-129</td>
</tr>
<tr>
<td>IL-6 RO XCTTGGAGGTCAGGGTCTTGTTTCG</td>
<td>340</td>
<td>185-189</td>
</tr>
<tr>
<td>IFNγ 5'-GGTCTGGTGCCTGATCTGACCC</td>
<td>579</td>
<td>165-169</td>
</tr>
<tr>
<td>IFNγ 3'-GTGGACATCAAGTCAGTTACC</td>
<td>185-189</td>
<td></td>
</tr>
<tr>
<td>IFNγ RO XTTGAGGAAATTGACGGATTTGTGCC</td>
<td>688</td>
<td>165-169</td>
</tr>
<tr>
<td>IL-β 5'-AAAAGTGATTGATGCAGTCTGCA</td>
<td>688</td>
<td>165-169</td>
</tr>
<tr>
<td>IL-β 3'-CTTGGGATGCTAAGGAGATCTCCT</td>
<td>579</td>
<td>165-169</td>
</tr>
<tr>
<td>β Actin 5'-TCTCGAGGGAGGAGCTGTAACGG</td>
<td>340</td>
<td>185-189</td>
</tr>
<tr>
<td>β Actin RO XAAGTACCTTGCCTGTCGGTCT</td>
<td>340</td>
<td>185-189</td>
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* Two sizes of run-off product are given for each cytokine which correspond to run-off reactions of either wild-type cDNA or DNA internal standard (Fig. 1). In all cases, the 5' and 3' primer spanned at least one intron in the genomic DNA sequence so that any amplification of contaminating genomic DNA would be easily identified.  
  
* X represents the fluorescent dye (Fam) which was conjugated as recommended by the supplier (Applied Biosystems).

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Since β-actin mRNA and IL-6 mRNA may present some differences with regard to their half-life and stability, we checked whether samples in which IL-6 gene expression was low (<5 IL-6 mRNA copies/10^6 β-actin mRNA copies) or undetectable expressed mRNA for other cytokines. In 12 of 13 cases, a significant expression of IL-1β or IFN-γ could be demonstrated in these samples after PCR, when analyzed on ethidium bromide-stained agarose gel (Fig. 3). In these same samples, very low or no expression of IL-6 could be visualized after PCR on ethidium bromide-stained agarose gel (data not shown). Moreover, a quantitative PCR assay for IFN-γ did not find any correlation between IFN-γ mRNA concentrations and the various groups of patients with invasive cervical carcinoma or CIN and normal cervix (Fig. 4).

Therefore both the presence of IL-1β and IFN-γ gene expression in samples with negative or low IL-6 gene expression and the absence of correlation between mRNA levels of other cytokines, such as IFN-γ and invasive cervical carcinoma, confirmed the relevance of IL-6 mRNA level distribution in the various groups of patients.

**Fig. 1.** Peak fluorescence of wild-type cDNA and DNA internal standard for IL-6, IFN-γ, and β-actin. For each parameter, internal standard and target cDNA were coamplified with the same specific primers. A run-off reaction with a third nested specific fluorescent primer was then carried out and the PCR product loaded on an automated sequencer. After electrophoresis, the fluorescent profiles were recorded. The peak area ratio between known concentrations of standard DNA and target DNA was used to determine concentrations of wild-type cDNA. Numbers correspond to the size of the run-off products.

**Fig. 2.** Comparative analysis of IL-6 gene expression in primary invasive cervical carcinomas, CIN, and normal cervix by quantitative PCR. Twenty cDNAs derived from mRNA extracted from cervical biopsy specimens were coamplified with internal standard for IL-6 or β-actin, with specific IL-6 or β-actin primers (Table 1). A run-off reaction with a third specific fluorescent IL-6 or β-actin primer was then performed. After electrophoresis, the fluorescent profile was recorded and peak areas were computed. Therefore, IL-6 mRNA copies can be normalized to a constant amount of β-actin mRNA copies.

**Fig. 3.** IL-1β and IFN-γ gene expression in tumor biopsies and normal cervix with undetectable or low IL-6 gene expression. Thirteen cDNAs derived from mRNA extracted from 6 primary invasive cervical carcinomas (Lanes 1–6), 5 CIN (Lanes 7–11), and 2 normal cervix (Lanes 12 and 13) were selected to contain less than 5 IL-6 mRNA copies/10^6 β-actin mRNA copies as measured by quantitative PCR assay. An equivalent amount of these cDNA normalized to 10^6 β-actin mRNA copies was amplified by using oligonucleotide primers specific for IL-1β (A) or IFN-γ (B).

**Fig. 4.** Comparative analysis of IFN-γ gene expression in primary invasive cervical carcinomas, CIN, and normal cervix by means of quantitative PCR. The same protocol described in the legend to Fig. 2 was used to measure IFN-γ mRNA expression in 16 cervical biopsy specimens.

**Analysis of IL-6 Immunostaining in Different Cervix Tissues.** IL-6 immunostaining was detected in 5 of 13 cervical carcinoma tissues previously fixed in formalin. Positive immunostaining for IL-6 was demonstrated in 5 of 10 cervical carcinomas with detectable IL-6 gene expression. We did not find any correlation between IL-6 immunostaining intensity and the level of IL-6 gene expression as detected by quantitative PCR. However, no immunostaining was seen in tissue samples in which IL-6 gene expression was undetectable.
Cells that stained for IL-6 were only present in the tumor stroma and no tumor cells were found to be positive (Fig. 5). Only double immunostaining could definitively identify these IL-6 positive cells but, based on morphological criteria, these cells seemed to belong to the macrophage lineage.

Correlation between Immune Cell Infiltrate and IL-6 Gene Expression. We therefore attempted to correlate IL-6 gene expression with CD68-positive monocytes and CD3-positive T cells present in tissue infiltrates. As shown in Table 2, a clear correlation was demonstrated between IL-6 gene expression and high CD68-positive monocyte infiltrates. Indeed, all patients with high CD68-positive monocyte infiltrates had detectable IL-6 gene expression, whereas only 27% (3/11) of patients with low CD68-positive monocyte infiltrates expressed the IL-6 gene (P < 0.007). However IL-6 gene expression varied considerably within the group with high macrophage infiltration. It is noteworthy that the only patient with CIN and high CD68-positive monocyte infiltrates expressed only 2 IL-6 mRNA copies/10^6 beta actin mRNA copies (Fig. 2). In contrast no significant correlation was observed between CD3 T cell infiltrates and IL-6 gene expression (Table 2).

A trend toward a higher frequency of CD68-positive monocyte infiltrates associated with invasive cervical carcinoma rather than with CIN or normal cervix was observed but was not statistically significant (data not shown).

HPV DNA Detection and IL-6 Gene Expression. Because the DNA of HPV is found in a large proportion of cervical carcinomas and because it has been shown that keratinocytes transfected with HPV secrete IL-6 (31), a possible association between HPV DNA and IL-6 gene expression was investigated. Although there appeared to be an increased frequency of IL-6 gene expression in the HPV-positive groups, it was not statistically significant (P > 0.05; Table 2). The number of cases was too small to investigate an association between a specific HPV type and IL-6 gene expression.

DISCUSSION

By means of a quantitative PCR technique previously developed in the mouse (23, 29), we found an increased IL-6 gene expression in primary invasive cervical carcinomas as compared to CIN or normal cervix. In contrast to other PCR quantitative techniques using either no internal standard (32) or an internal standard not homologous to the DNA target (33, 34), the advantages of this technique consist of the almost identical nature of the standard and target DNA. Therefore, because the wild-type and standard cDNA underwent the same variations during their coamplification, their ratio remained constant in the various experiments and bias due to a change in amplification efficiency of different samples was avoided. Since all our results were normalized to a constant number of beta actin mRNA copies, differences in sample integrity or RNA extraction and cDNA synthesis efficiencies cannot account for the difference in the distribution of IL-6 gene expression in the two groups of patients. Moreover, we showed that, unlike IL-6, levels of IFN-gamma mRNA expression were not correlated with specific cervical histological lesions (Fig. 4).

Immunohistochemical analysis identified IL-6 protein only on stromal cells, which on the basis of morphological criteria, most likely belong to the macrophage lineage. This was reinforced by the correlation observed between IL-6 gene expression and macrophage infiltration (Table 2). No IL-6 immunostaining of cervical tumor cells was demonstrated. This does not preclude that tumor cells expressed some IL-6 mRNAs which were not translated.

In some cases, we could not detect IL-6 protein, but found IL-6 gene expression using PCR; this could reflect either a low translation of IL-6 gene in some circumstances, a lower sensitivity of immunocytochemistry, or the loss of IL-6 epitopes during certain fixative procedures. However, the formalin fixation procedure has already been used for IL-6 immunostaining with satisfactory results (35).

This in vivo localization of IL-6 may seem contradictory with in vitro studies showing IL-6 secretion by cervical tumor cell lines (22). However, a down-regulation of IL-6 secretion has been reported in cervical cell lines immortalized by recombinant HPV16 (36). Discrepancies in cytokine expression between cells in vivo and in vitro are also frequently observed (37), which reinforces the necessity of techniques allowing quantification of gene expression in vivo.

In renal cell carcinoma (38) or glioblastoma (17), synthesis of IL-6 by tumor cells was demonstrated in vivo, suggesting possible autocrine effects of IL-6, whereas in ovarian carcinoma (18, 39) or myeloma (40), a paracrine effect of IL-6 was reported since no IL-6 secretion by tumor cells could be observed. It is noteworthy that TAMs from ovarian carcinoma constitutionally release high levels of IL-6, whereas low levels of IL-1 or tumor necrosis factor are produced by TAMs in cancer patients (39, 41). This could apply to cervical carcinomas, in which, apart from dysregulation of IL-6 gene expression, other cytokine gene expressions did not seem to be overexpressed in these tumors (Fig. 2 and data not shown).

It was recently shown that IL-6 secretion may be a feature more frequently associated with metastatic melanoma cell lines rather than primary melanoma cells (42). It is of interest that we only studied primary invasive cervical carcinomas. Increased IL-6 expression in tumor tissue does not appear to be a general phenomenon, since Basolo et al. (43) did not find IL-6 immunostaining in 13 patients with breast cancers, whereas normal mammary epithelial cells release IL-6. Similarly, Pisa et al. (44) did not observe increased IL-6 gene expression in ovarian tumors as compared to normal ovaries.

The significance of this high IL-6 mRNA concentration in cervical tumors remains to be determined.

Table 2. Relationships between level of IL-6 gene expression and monocyte or T-cell infiltrate and presence of HPV genome

<table>
<thead>
<tr>
<th></th>
<th>IL-6 &gt; 1 copy</th>
<th>IL-6 &lt; 1 copy</th>
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<tbody>
<tr>
<td>Low monocyte infiltrate</td>
<td>3/11</td>
<td>8/8</td>
</tr>
<tr>
<td>High monocyte infiltrate</td>
<td>8/11</td>
<td>0/8</td>
</tr>
<tr>
<td>Low T cell infiltrate</td>
<td>2/11</td>
<td>5/8</td>
</tr>
<tr>
<td>High T cell infiltrate</td>
<td>9/11</td>
<td>3/8</td>
</tr>
<tr>
<td>Presence of HPV genome</td>
<td>10/12</td>
<td>4/8</td>
</tr>
<tr>
<td>Absence of HPV genome</td>
<td>2/12</td>
<td>4/8</td>
</tr>
</tbody>
</table>

* Monocytes and T cells were identified by positive immunostaining with anti-CD68 antibody. Paraffin-embedded sections fixed in formalin were stained with polyclonal anti-CD68 antibody according to the LSAB technique (see "Materials and Methods").
* Presence of HPV genome was determined by PCR on formalin-fixed paraffin-embedded sections (33).
* All IL-6 mRNA quantification was performed on 10^6 beta actin mRNA copies.
cancers seems rather complex. Indeed, \textit{in vitro}, IL-6 can act as a growth factor for cervical carcinoma cell lines (22). If thrombocytosis, frequently observed in cervical carcinoma with poor survival, reflects IL-6 secretion, then high levels of IL-6 mRNA may indicate a poor prognosis (20). In other tumors, such as breast cancer, IL-6 may promote tumor metastasis and invasion by increasing motility and decreasing adherence of breast carcinoma cell lines (45). Alternatively, IL-6 is also able to inhibit the growth of human breast, leukemia/lymphoma, and primary melanoma cell lines (46, 47). Finally, in some studies, no significant growth inhibitory or stimulatory effect of IL-6 could be demonstrated on various human cell lines derived from a wide range of solid tumors (48).

The action of IL-6 on the immune system may be beneficial, since IL-6 released by HPV type 16 harboring keratinocytes mediated tumor death by increasing natural killer cell activity (31). Moreover, impaired immune function of macrophages against intracellular bacteria was demonstrated in IL-6-deficient mice (49). Conclusions drawn from experiments with tumors transfected with IL-6 cDNA, in order to induce antitumor immunity, have been rather contradictory (50, 51). Similarly, the role of TAM, a potential source of IL-6 in our study, is viewed as a balance between promoting functions on tumor cells, e.g., growth factor release and angiogenesis, and inhibitory activities (52). Prehn (53) recently pointed out that the inhibitory or stimulatory effect of immune reactions against tumor cells may depend on the level of immune reactions. In a model of fibroblasts genetically engineered to secrete IL-12, it was shown that low doses of IL-12 can induce a greater degree of antitumor immunity than high doses (54). These few examples emphasize the potential value of accurately quantifying immune parameters, i.e., cytokine, at the tumor site.

Our study confirms, \textit{in vivo}, that IL-6 may play a role in the pathogenesis of carcinoma of the uterine cervix, since its increased expression is associated with advanced neoplastic cervical lesions. The quantitative PCR assay used also revealed the marked variation of IL-6 gene expression from one sample to another, since the number of IL-6 mRNA copies may vary by a factor of log 6. Studies are currently underway to assess the prognostic value of this enhanced IL-6 gene expression in invasive cervical carcinomas.

To our knowledge, this analysis of IL-6 gene expression is the first report of quantitative cytokine gene expression in tumor tissue, since Yamamura et al. (55) used a semiquantitative PCR assay to determine cytokine profile in melanoma biopsies. Many studies have already emphasized the prognostic value of serum cytokine level in cancer patients (56). Because of their short half-life in serum and some bias introduced by various factors, determinations of cytokine mRNA concentrations using quantitative PCR may help to define their role in various tumors.

REFERENCES


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