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1 Cervical Cancer: pathogenesis

1.1 Incidence and Epidemiology

Cervical cancer is the second most common cancer amongst women world-wide with an incidence of around 500,000 new cases each year [1]. Developing countries account for 80% of cervical cancer cases [1,2]. The incidence rates vary among geographical areas and depend on factors like early age at sexual initiation, high number of sexual partners, high frequency of exposure to other risk factors (e.g., smoking, other sexually transmitted diseases and poor hygiene) and especially access to routine screening programs [3]. In developed countries, the incidence of cervical cancer is markedly reduced because of the introduction of large-scale cytological screening programs that allow detection and treatment of premalignant disease.

1.2 Classification

Cervical cancer generally originates in the transformation zone, the area of the cervix where the ectocervix and endocervix meet. Carcinomas of the cervix can be classified into squamous carcinomas, adenocarcinomas, adenosquamous carcinomas and neuro-endocrine tumors [4]. About 80% of epithelial cervical tumors are squamous cell carcinomas [5]. The development to cervical cancer is a multi-step process which commences with precursor lesions. The preneoplastic dysplastic changes of squamous carcinoma are referred to as cervical intraepithelial neoplasia (CIN). CIN is divided into three grades, known as CIN1, CIN2 and CIN3. These lesions are classified histologically on the basis of the proportion of epithelial cells with atypia that increases with grade. Most instances of CIN do not progress to invasive carcinoma, probably because human papillomavirus (HPV) infections and CIN are cleared by the immune system [6,7]. Only 1% of cases with CIN1, 5% of cases with CIN2 and 12% of cases with CIN3 will ultimately progress to cancer [8]. Adenocarcinomas represent 10-15% of cervical tumors. These have a glandular origin and their precursor lesion is referred to as cervical glandular intra-epithelial neoplasia (CGIN) and adenocarcinoma in situ. Staging of cervical carcinomas is based on clinical evaluation using the ‘International Federation of Gynecology and Obstetrics’ (FIGO) classification system.

1.3 Carcinogenesis

Human Papillomavirus

Persistent infection with high-risk HPV is the basic cause of cervical cancer [9,10]. More than 150 different HPV genotypes have been defined including 40 anogenital types, at least 13 of these are oncogenic [11,12]. HPV 16 and 18 are the most common HPV types in cervical cancer.

Infection with HPV starts when the virus has gained access to the keratinocytes in
the basal cells of the cervical epithelium. For its life cycle, HPV depends on the
differentiation of the keratinocytes. The expression of 3 viral proteins, E5, E6 and E7,
occur as the cervical epithelial cells go through differentiation. These viral early
proteins stimulate cell cycle reentry, to guarantee a DNA replicative machinery, on
which the virus relies for replication of its genome. E6 and E7 viral proteins interfere
with the function of p53 and proteins of the retinoblastoma family. These proteins
control cell cycle proliferation and represent important tumor suppressor genes [13].
By deregulating the normal cell cycle, the virus initiates premalignant lesions. The
HPV DNA is usually extrachromosomal in benign cervical precursor lesions. The
integration of HPV DNA into host-cell DNA of proliferating cells, leading to increased
stability of E6 and E7 viral transcripts [14], is likely to be a critical event in cervical
carcinogenesis [15].

Genetic factors

Only a small fraction of women with HPV infection in the transformation zone will
ultimately develop cervical cancer, indicating that other factors are involved in
carcinogenesis. Cervical tumors are characterized by complex genetic alterations
throughout the genome. These might involve inactivation of tumor suppressor genes,
resulting in loss of function, and amplification or activation of oncogenes, resulting in
a gain of function. The most common chromosomal regions with amplification are 1q,
3q, 5p and 8q. Candidate oncogenes in these regions are PI-3 kinase/AKT (3q), c-
Myc (8q) and TERT (5p). These genes are involved in cell growth and survival and
become activated when amplified [16–18]. Loss of genetic material occurs frequently
at chromosomal region 2q, 3p, 4p, 4q, 5q, 6p, 6q, 11q, 13q, and 18q [19]. Possible
tumor suppressor genes in these chromosomal regions are FHIT (3p) [20], involved
in cell cycle control and apoptosis, NOL7 (6p), possibly involved in inhibition of
angiogenesis [21], and human leukocyte antigens (HLA) class I (6p), involved in
antigen presentation [22]. Inherited susceptibility to cervical cancer may have some
influence. Families in which more than one woman has cervical cancer are rare, although
there is evidence that having a sister or a mother with cervical cancer increases a
woman's risk of cervical cancer two-fold [23].

Factors associated with the immune system

Variations in the capacity to clear an HPV infection by an effective immune response
can be one explanation why certain HPV infections give rise to lesions. The importance
of an adequate immune response to prevent the development of cervical cancer is
underlined by observations that immunosuppressed women, HIV/AIDS patients [24]
and recipients of organ transplants [25,26], show an increased risk of cervical cancer.
This implies that a reduced risk of cancer would be expected in women with increased
immunologic activity, e.g. allergic individuals. Indeed, for women having sons with
allergy (rhinoconjunctivitis, asthma or eczema) the risk of cervical cancer is decreased
[27]. Particular alleles involved in immune responses may confer protection or
susceptibility to cervical cancer, such as the genes for ERAP1, TAP2 and LMP7,
involved in antigen processing [28]. Also, certain alleles of the polymorphic HLA class I and II genes of the major histocompatibility complex (MHC), involved in antigen presentation, seem to display protection or susceptibility in terms of high-grade CIN development [29,30]. Carriers of commonly reported protective HLA class II alleles displayed lower viral load, short-term HPV infection and a decreased risk of cervical carcinoma in situ [30].

2 The immune system

2.1 Immune response

The immune system consists of an innate and acquired branch. Innate immunity is non-specific and functions as a first line of defense. There are different types of defensive barriers within the innate immunity: anatomy (e.g. skin and mucus); physiology (e.g. temperature and pH); humoral factors, such as the complement system and cells (monocytes, neutrophiles and macrophages) that can phagocytose (ingest) particles or microorganisms. The acquired immune system is activated by the innate immune system and is capable of recognizing and remembering specific antigens. Thereby it protects the body against the clinical manifestations of subsequent infections with the same microorganisms. The acquired immune system is represented by a cellular and a humoral section, in both sections lymphocytes play a central role. Humoral immunity is mediated by antibodies, secreted by plasma cells (differentiated B lymphocytes). Recognition of an antigen by a receptor molecule on its surface, leads to activation of B lymphocytes and the generation of plasma cells. By binding antigens of foreign agents (e.g. bacteria), antibodies flag these for destruction [31]. Antibodies can also neutralize toxins or viruses by preventing adhesion of these pathogens to host cells. T lymphocytes, in which T helper cells and cytotoxic T lymphocytes (CTL) are identifiable sub-groups, play a central role in cell-mediated immunity. Recognition of a foreign antigen occurs by a T-cell receptor in association with antigen-presenting proteins, called HLA. The HLA proteins act as "signpost" that display fragmented pieces of an antigen (peptides), either self or nonself, on the cell's surface. This enables the immune system to distinguish between normal and abnormal cells [32]. Antigen can be presented either by HLA class I or HLA class II proteins. HLA class I is expressed on most cell types whereas HLA class II is expressed predominantly on antigen presenting cells (APC) such as B lymphocytes, dendritic cells and activated macrophages. T helper cells recognize antigen in context of HLA class II proteins. Upon activation, T helper cells release cytokines that influence the activity of other cell types such as B lymphocytes, CTL and phagocytic cells. Therefore, T helper cells play a central role in the regulation of innate and acquired immunity. CTL recognize antigen in context of HLA class I proteins. Recognition occurs by presentation of foreign peptides in the context of HLA class I displayed on cells. Subsequently, CTL kill these cells by releasing cytotoxins that form pores in the cell membrane, inducing activation of an intracellular suicide mechanism, a process
called apoptosis [33]. Thus a cell-mediated immune response is essential for killing of virus-infected cells, intracellular bacteria, fungi and cancer cells.

Besides recognition of antigen, a second signal is usually required for activation of B- and T lymphocytes. In case of T lymphocytes, co-stimulation occurs through engagement of CD28 with B7 molecules on APC [34]. Cytokines, secreted by activated T helper lymphocytes, are co-stimulatory signals necessary for growth and differentiation of B lymphocytes and CTL. In the absence of co-stimulation, anergy, the inability of immune cells to mount an adequate immune response against an antigen, can occur. Different T helper cell subsets, designated Th1, Th2 and Th17 can be distinguished by the cytokines they secrete (Fig. 1). Th1 cytokines include IFNγ that activates CTL, thereby stimulating cell-mediated immunity. In contrast, Th2 cytokines, such as IL-4, IL-5, and IL-10, function as a helper for B lymphocyte activation. A Th2 response is often elevated in allergic disease and parasitic infections [35]. Th17 is a recently discovered T cell subset that is involved in the control of infections against some extracellular bacteria [36,37]. The characteristic cytokine expressed by Th17 cells is IL-17. IL-17 mediates a pro-inflammatory response by inducing the expression of other cytokines (e.g. IL-6) and chemokines (e.g. IL-8) [38]. Furthermore, other subpopulations of T lymphocytes, named suppressor or “regulatory” T lymphocytes can suppress the immune system by producing inhibitory cytokines such as IL-10 and TGF-β. T lymphocytes can regulate the intensity of an immune response by switching from immune-promoting cytokines (e.g. IFNγ) to inhibiting cytokines (IL-10) in a later stage of the response.

![Diagram of cytokines involved in differentiation of T helper lymphocyte subsets](image)

**Figure 1.** Cytokines involved in differentiation of T helper lymphocyte subsets. Abbreviations: DTH, delayed type hypersensitivity; Ab, antibodies. From Chabalgoity et al., 2007 [35].
2.2 Immune response in cervical cancer

HPVs do not provoke an adequate humoral immune response. Naturally arising antibodies against HPV do not seem to be effective in preventing subsequent infections of homologous or genetically related HPV types [39]. On the contrary, antibodies against HPV capsid proteins that arise after vaccination can be protective at preventing infections of homologous or genetically related HPV types [40,41]. Nevertheless, antibodies do not play an important role in the regression of established HPV infections and cervical lesions [42]. Cellular immune responses however are likely to be an important effector mechanism for the clearance of established infections [43]. HPV-specific T cell immunity is frequently detected in healthy individuals. Presence of T cell immunity therefore plays a role in protecting against persistent HPV infection and hence in the development of cervical malignancies [44]. In patients with cervical lesions and cervical cancer, tumor-specific T lymphocytes against the two HPV encoded oncoproteins, E6 and E7, were detected at low levels in the peripheral blood of approximately 50% of the patients [45-47]. HPV-specific systemic immunity in patients may not be beneficial since it has been associated with a non-inflammatory cytokine profile and did not correlate with prognostic factors [47,48]. In contrast, presence of high numbers of tumor-infiltrating lymphocytes has been associated with a survival advantage in patients with cervical cancer [49,50]. However, recent data suggests that the ratio between infiltrating CTL and infiltrating regulatory T lymphocytes is also an important determinant for prognosis [48],[51]. By suppressing proliferation and cytokine production of CTL, these regulatory T lymphocytes may well interfere with the tumor-specific immune response [52].

3 Immune escape

Due to the presence of foreign proteins of viral origin, and the higher incidence of cervical cancer in immunosuppressed women, cervical cancer is considered an immunogenic cancer. However, HPV have developed a number of immune evasive mechanisms and are therefore poor stimulators of the immune system [53]. HPVs minimize exposure to the immune system by executing viral replication and assembly only in a fully differentiated cell. Consequently, there is no opportunity for APC to engulf virions and present virus-derived peptides to the immune system. Also, there is no blood-borne phase of infection. HPVs do not infect and replicate in APC, nor lyse keratinocytes and therefore do not elicit any pro-inflammatory signals [54]. Furthermore, unlike APC, keratinocytes have low levels of HLA class I and class II molecules, and lack ‘co-stimulator’ molecules such as B7 [55]. The absence of costimulatory and proinflammatory signals is detrimental for the development of an effective immune response against HPV and may result in anergy. Not only do HPV early proteins interfere with cell signaling, targeting genes involved in cell cycle or apoptosis, but also interfere with antigen presentation and expression of cytokines [56]. During the development and progression of cervical cancer also alterations in the cell on (epi)genetic level, influence immune evasion. Two important mechanisms
will be discussed more in detail in the next 2 paragraphs.

3.1 Alterations in HLA class I expression

An important mechanism of immune escape in cervical cancer is HLA class I loss. HLA class I comprises a highly polymorphic heavy chain, encoded by a HLA-A, HLA-B or HLA-C genes located on chromosome region 6p21.3 and a light chain, β2-microglobulin. The extracellular part of the heavy chain contains a peptide-binding groove, in which peptides bind in an allele-specific manner. Since HLA molecules are responsible for the presentation of foreign antigens, these molecules play a central role in the immune recognition and subsequent clearance of virus-infected or other deviating cells [57]. Thus loss of HLA class I enables tumor cells to evade recognition and lyses by CTL. Especially in case of tumors with a viral origin such as cervical-cancer, development of a powerful immune response could be hindered since viral antigen is not or insufficiently presented. Defects in HLA class I are caused by multiple mechanisms [58]. Total HLA class I loss can be the result of mutations in the β2-microglobulin-gene or defects in the molecules of the HLA class I processing and presentation pathway such as peptide transporter TAP1 or TAP2. Low expression of TAP1 and TAP2 have been observed in cervical cancer cell lines and tumors [59,60]. Partial HLA class I loss can be explained by (structural) defects in the particular HLA proteins which can be caused by locus-specific downregulation, possibly owing to shortcomings at the transcriptional level. Also loss of a single class I allele by mutations or deletions and loss of HLA class I haplotype by loss of heterozygosity (LOH) can be the cause for partial HLA class I loss. LOH can be due to mitotic recombination, deletion or chromosomal nondisjunction [58]. A detailed study of HLA class I aberrations in cervical cancer showed that approximately 90% of the cervical tumors had HLA class I defects [22]. The major mechanism for loss of expression was LOH (50%). Also HPV might interfere with HLA expression as a mechanism to escape from host immune surveillance. HPV16 E5 was shown to impede transport of HLA class I complexes to the cell surface and downregulate surface expression of HLA-A and HLA-B [61]. The importance of HLA class I expression in cervical cancer is emphasized by the findings that reduced expression of HLA class I correlates with worse prognosis and overall survival [62,63].

3.2 Immunosuppressive cytokines

Due to their central role in the immune system, cytokines are involved in a variety of immunological and inflammatory diseases, including cancer. In general, cytokines do not function individually but in concert and have pleiotropic and redundant functions. Th1 cytokines are proinflammatory and boost the immune response, whereas Th2 cytokines, which play a role in humoral immunity, are immune-inhibitory towards cell-mediated immunity. Th1 cytokines are indispensable for the generation of an adequate anti-tumor immune response [64]. However, continuous expression of Th1 cytokines such as TNFα can result in a chronic inflammatory process which might promote cancer [65]. Acute inflammation contributes to the regression of cancer whereas chronic
Chapter 1

inflammatory diseases are frequently associated with increased risk of cancer [64,66,67]. During a chronic inflammatory process, besides many cytokines (including growth and angiogenic factors), reactive oxygen and nitrogen species are generated that may cause DNA damage and predispose to neoplasia [67]. In the process of cancer development, cytokines produced by tumor cells, immune cells or stroma, facilitate cancer growth [68]. However in immunogenic cancer types, vaccination with tumor cells in combination with proinflammatory cytokines, including TNFα, IFNγ, IL-2 and GM-CSF, does show therapeutic benefit [69-72]. Evidence exists that this is associated with anti-tumor activity by T lymphocyte-mediated responses [72]. The contribution of proinflammatory cytokines to the initiation and development of cancer or prevention of cancer and anti-tumor activities depends on factors like the type of cancer, the tumor stage and the tumor micro-environment. In cervical cancer, several studies provided evidence that the cause of a failing anti-HPV immune response was a decrease in proinflammatory cytokines such as TNFα [47,73-75]. Also, several studies reported a predominant Th2 cytokine pattern in cervical lesions and cancer [73,76]. Polarization towards a Th2 cytokine profile signifies downregulation of a proinflammatory immune response and therefore a decreased susceptibility to the effect of CTL.

Expression of immunosuppressive cytokines like IL-10 and transforming growth factor β (TGF-β) is another strategy used by tumors to escape immunosurveillance [77]. These cytokines inhibit the function of CTL and promote a shift towards Th2 cytokine expression, thereby downregulating anti-tumor immunity [76,78,79]. Tumor-derived IL-10 and TGF-β have been shown to induce regulatory T lymphocytes and skew the immune response to Th2 polarity in cervical cancer [76]. It is conceivable that cervical cancers circumvent the development of a potent HPV-specific anti-tumor immune response by not providing the appropriate proinflammatory environment and instead stimulate the expression of immunosuppressive cytokines as TGF-β and IL-10. TGF-β will be discussed in more detail in the next chapter, since a substantial part of this thesis concerns research into this cytokine.

4 TGF-β

TGF-β is a pleiotropic cytokine that apart from its immunosuppressive function, is involved in the regulation of many biological processes including cell growth, development, apoptosis, differentiation, migration, angiogenesis and extracellular matrix production. Three isoforms of TGF-β exist (TGF-β1, TGF-β2 and TGF-β3), which belong to the TGF-β superfamily that among others include activins and bone morphogenetic proteins. In a large number of human cancers, changes in TGF-β signaling are frequently observed [80]. TGF-β signaling can have both positive and negative effects in cancer depending on the cell type, stage of carcinogenesis and the responsiveness of the tumor cell.
4.1 Signal transduction

TGF-β1 is secreted as part of a latent complex into the extracellular matrix [81]. "TGF-β activators" such as thrombospondin, matrix metalloproteinases, plasmin and certain integrins release TGF-β1 from its latent state [78, 82]. Once activated, TGF-β family members initiate signaling by interacting with receptor serine/threonine kinases, type I and type II (Fig. 2). Upon ligand binding, the TGF-β receptor type II (TβRII) phosphorylates the TGF-β receptor type I (TβRI) which leads to the activation of its kinase domain and subsequent regulation of the mothers against decapentaplegic drosophila homolog (Smad) proteins. Activated TβRI phosphorylates Smad2 and Smad3 proteins, which form hetero-oligomeric complexes with Smad4. These complexes translocate to the nucleus and, depending on the cell type and their

Figure 2. TGF-β1 induced activation of Smad and MAPK and their interactions. Activation of Smad signaling occurs once TGF-β1 binds to the TGF-β receptor type II (TβRII). Subsequently, TβRII phosphorylates TGF-β receptor type I (TβRI) which in turn phosphorylates Smad2/3. Then, activated Smad2/Smad3 bind Smad4 and translocate to the nucleus where the complexes function as transcription factors. Transcription is controlled by the presence of coactivators (Co-A) or corepressors (Co-R). Activation of the 3 MAPKs (ERK, JNK and p38) by TGF-β1 or other stimuli such as growth factors or proinflammatory cytokines, can regulate Smad activation by direct phosphorylation or through downstream effector molecules. Examples of downstream effector molecules are c-Jun and ATF2 which modulate transcriptional activity. From Javelaud et al., 2005 [84].
interactions with certain transcription factors, activate or repress transcription via
direct DNA binding [83].

Evidence exists for a strong integration of Smad signaling within a complex network
with other signaling pathways that largely contribute to modifying the initial Smad
signals and allows diverse responses to TGF-β1 [84,85]. Smad signaling may even
be dispensable for some of the responses initiated by TGF-β1 [86,87]. TGF-β1 has
been shown in various cell types to activate extracellular signal-regulated kinase
(ERK), p38 and Jun kinases (JNK) which are mitogen activated protein kinases (MAPK)
[88,89]. ERK-mediated pathways are involved in proliferation and usually anti-apoptotic.
In contrast, JNK and p38-signaling, activated by stress stimuli, often induce
apoptosis. Activation of MAPK by TGF-β1 can occur directly by TGF-β activated
kinase-1 (TAK1) or indirectly, possibly resulting from Smad-dependent transcriptional
responses [90]. MAPK can positively and negatively influence TGF-β1 signaling [84].
MAPK negatively influences TGF-β1 signaling by stimulating expression of Smad7,
an inhibitor of Smad signaling [91]. Furthermore, phosphorylation of the linker region
of R-Smads may block nuclear translocation and signaling by Smads [92]. MAPK are
positively implicated in TGF-β1 signaling by interaction of AP-1 transcription factors,
downstream components of the MAPK pathway, with Smad complexes to regulate
transcription [93]. Also other signaling pathways, including the PP2A/p70S6, Rho
GTPases, PI-3 kinase/AKT and Wnt pathways, can either be induced by TGF-β1, or
can modulate the outcome of TGF-β-induced Smad signaling [94-97].

4.2 Role in cancer

TGF-β1 as a tumor suppressor

During tumor initiation and early progression TGF-β1 is thought to be a tumor
suppressor, whereas late in tumor progression TGF-β1 signaling promotes
tumorigenesis (Fig. 3). The anti-tumor response by TGF-β1 early in tumorigenesis is
mediated by its growth inhibiting and pro-apoptotic properties via both Smad and
non-Smad signaling pathways [85]. TGF-β1 stimulation of epithelial cells has been
shown to induce a G1 cell cycle arrest by activating various anti-proliferative responses
such as the transcriptional upregulation of cyclin-dependent kinase (CDK) inhibitors
p15 and p21 [98,99]. Also, TGF-β1 transcriptionally represses cdc25A [100], a
phosphatase that removes inhibitory phosphatidyl groups from CDK, the progrowth
transcription factor c-Myc and the inhibitors of differentiation Id1, Id2 and Id3 [101,102].
Pro-apoptotic responses by TGF-β1, often mediated by Smad proteins, are caspase-
8 activation via upregulation of the FAS receptor, transcriptional induction of death
associated protein kinase and upregulation of the pro-apoptotic proteins Bim and
Bmf [103-105]. In view of the above, somatic mutations and loss of expression of
various components of the TGF-β-Smad signaling pathway, could lead to resistance
of TGF-β1 mediated growth inhibition in cells. In pancreatic cancer, Smad4 is
genetically inactivated in approximately 50% of tumors [106]. Also in certain colorectal
cancers, Smad4 mutations are often found [107]. TBR1 mutations are frequently found
in cancers associated with microsatellite instability, such as hereditary non-polyposis
colorectal cancer [108]. Mutations in TβRI and Smad2 occur at low frequency, whereas Smad3 mutations have not been detected so far [80]. Loss of expression of these components was often found to be associated with worse prognosis [109,110]. However, some alterations of the TGF-β1 pathway do not lead to a complete loss of signaling and could result in loss of tumor suppressive effects while retaining the tumor promoting effects [80]. An alternative way of inactivating the tumor suppressor function of TGF-β1 is by overexpressing or amplification of its inhibitors. For example, Smad7 amplification in colorectal cancer and high expression of SMURF2 in esophageal squamous cell carcinoma were found to be poor prognostic markers [111,112]. The proto-oncogene product Ski was shown to promote tumor growth in pancreatic cancer by abrogation of TGF-β1 signaling [113]. Furthermore, oncoproteins c-Myc and Ras were shown to antagonize the growth-inhibitory response of TGF-β1 signaling in epithelial cells [92,114-116].

**Figure 3.** TGF-β1 switches from tumor suppressor in normal and premalignant stages of tumorigenesis to proto-oncogene at later stages of disease. Progression to metastatic disease is usually accompanied by decreased or altered TGF-β1 responsiveness and increased expression/activation of TGF-β1. Besides changes in the responsiveness to TGF-β1 in tumor cells, TGF-β1 effects tumor stroma in such a manner that tumor growth and metastasis is facilitated. From Roberts et al., 2003 [117].

**TGF-β1 as a tumor promoter**

During the process of carcinogenesis, cells often lose the anti-proliferative or apoptotic response to TGF-β1 and show increased production of this cytokine. The tumor-promoting role of TGF-β1 is mediated through its effect on tumor cell invasion, angiogenesis, changes in the tumor microenvironment and the cells of the immune system. TGF-β1 can induce an invasive phenotype in cancer cells, characterized by
an epithelial-to-mesenchymal transition (EMT) [118,119]. Cells undergoing EMT exhibit changes in morphology, loose cell-cell and cell-matrix adhesive properties accompanied by degradation of the surrounding extracellular matrix (ECM). A number of studies have shown that the Smad pathway as well as other pathways in TGF-β1 signaling such as ERK and PI-3 kinase/AKT signaling are required for EMT [120-123]. Increased production and activation of TGF-β1 by tumor cells stimulates synthesis of ECM proteins and chemokine production as well as activation of fibroblasts [124,125]. By providing growth factors, ECM proteins and matrix metalloproteinases, fibroblasts support tumor progression [126,127]. Furthermore, TGF-β1 stimulates tumor angiogenesis. Angiogenesis is crucial for tumor growth and invasion, as blood vessels deliver nutrients and oxygen to the tumor cells and allow migration of tumor cells via the blood system, resulting in metastasis. TGF-β1 induces expression of vascular endothelial growth factor (VEGF) [128], thereby stimulating proliferation and migration of endothelial cells [129] and capillary formation of endothelial cells [130]. In addition, TGF-β1 possesses chemotactic activity for monocytes which release angiogenic cytokines. Escape from immunosurveillance is mediated by inhibition of T lymphocyte proliferation through expression of cell cycle regulators and blockade of the production of IL-2, a cytokine known to activate T lymphocytes, NK cells and other types of the immune system [131]. TGF-β1 also controls T- and NK-cell effector functions by attenuating the cytolytic activity of these cells, inhibiting expression of IFN-γ, necessary for the stimulation of a Th1 response, and inhibiting the expression of perforin [132-134]. Furthermore, antigen presentation by differentiating dendritic cells, which are powerful APC, is hampered by inhibiting the expression of HLA class II and costimulatory molecules [135].

4.3 TGF-β1 in cervical cancer

During cervical carcinoma development, a progressive loss of sensitivity to TGF-β1-mediated growth inhibition was found [136]. This seems to be accompanied by increased expression levels of TGF-β1 during the progression from benign to malignant lesions [137-139]. Whereas TGF-β1 did not hinder proliferation of cervical cancer cells [138], an inverse relationship was found between its expression and the amount of tumor infiltrate [140]. Furthermore, TGF-β1 mRNA expression correlated with the amount of intratumoral stroma [140]. Consequently, the tumorsuppressive characteristics of TGF-β1, such as growth inhibition, seem to be lost, whereas the tumor-promoting features of TGF-β1, such as immunosuppression and ECM production, appear to be fully functional in cervical cancer. Mutations in signaling components of the TGF-β1-Smad pathway have been observed in cervical cancer and may play a role in carcinogenesis [141-143]. So far, mutations in Smad4 and TβRII were mainly found in cell lines [141,143,144]. A possible relation between mutation of Smad4, accompanied with loss of protein expression, and resistance to TGF-β1-induced growth inhibition, remains to be elucidated since data are conflicting [141,144]. Interference with TGF-β1 signaling can also occur through HPV E7 oncoproteins which bind Smad proteins and inhibit Smad-induced transcription [145,146]. HPV16 E7 may also interfere with TGF-β1 signaling by blocking TGF-β1
suppression of c-myc transcription via pRb. This has been observed in human foreskin keratinocytes transformed with HPV16 and was associated with resistance to the growth inhibitory effects of TGF-β1 [101]. However, TGF-β1 itself has been shown to inhibit expression of HPV16 E6 and E7, accompanied with cessation of cell proliferation in immortalized genital epithelial cell lines [147]. After malignant transformation, cells became partially resistant to the inhibitory effects of TGF-β1 on cell growth and gene expression of HPV16 E6 and E7 [147]. Thus far, it is unknown whether resistance to TGF-β1-induced growth inhibition is due to mutations in the Smad signaling pathway, interference by HPV oncoproteins or changes in signal transduction due to crosstalk with other pathways. Further insights into the signaling pathways downstream of TGF-β1, and the contributions of these pathways to the specific cellular and context-dependent effects of TGF-β1, may lead to more specific targeting of this pathway in the treatment of cancer.

5 Outline of the thesis

Cervical cancer is a virus-induced tumor that has developed several escape mechanisms to avoid eradication by the immune system. In this thesis we have investigated several of the immune escape routes that cervical cancer cells have acquired. A common feature of cervical cancer cells, occurring in ≥ 90% of tumors, is their ability to prevent recognition by the immune system through the loss of HLA class I protein. We hypothesized that tumors without HLA class I loss have developed other ways to prevent elimination by the immune system. In these latter cases, expression of immunosuppressive cytokines, such as TGF-β1 or IL-10, could be a mechanism to escape immune surveillance. In chapter 2, tumors positive and negative for HLA class I were compared, based on gene expression profile, to investigate escape routes in tumors with HLA class I expression. Microarray was used as a platform to study genomewide gene expression changes between tumors positive and negative for HLA class I. Subsequently, real-time PCR and immunohistochemistry were used to confirm expression changes. An alternative mechanism for immune escape in cervical cancer, besides loss of HLA class I and expression of immunosuppressive cytokines, is loss of proinflammatory cytokine expression. The presence of the proinflammatory cytokine TNFα may also be detrimental for the development of an adequate immune response against HPV.

Therefore, in chapter 3, loss of TNFα gene expression, as a mechanism to escape immune clearance, was investigated. The (epi)-genetic characteristics associated with the loss of TNFα expression were studied to investigate if TNFα could be a candidate tumor suppressor gene.

TGF-β1, a cytokine with potent immuno-inhibitory qualities, is excessively produced by cervical cancer cells. As discussed earlier, TGF-β1 suppresses the immune response by several mechanisms. However TGF-β1 also suppresses cell growth, therefore cervical cancer cells need to first acquire insensitivity to TGF-β1 induced cell growth inhibition, thus providing the tumor cells a growth advantage. In chapter 4, the gene expression profile (using microarray) of cervical cancer cell lines with a
different sensitivity towards TGF-β1 was investigated. To investigate if TGF-β1
insensitivity in cervical cancer can be due to alterations in the canonical Smad pathway,
loss of Smad2, Smad2-P and Smad4 protein expression was investigated by
immunohistochemistry in a large cohort of clinical samples as described in chapter
5. Tumors with low expression of Smad2-P and Smad4 were investigated for genetic
inactivation, loss of heterozygosity and in case of Smad4 for promoter methylation to
determine if these genes are candidate tumor suppressor genes in cervical
carcinogenesis.

Cervical tumors are characterized by complex genetic alterations involving deletions
and amplifications that can affect many chromosomal regions. Also genes involved
in immune responses can be affected, as has been shown for HLA class I genes.
Currently, large-scale DNA copy number, genotyping and gene expression profiling
methods are available to study the genome. To obtain an overview of genome-wide
molecular alterations in cervical cancer and to uncover genes involved in cervical
carcinogenesis, including genes related to the immune response, we investigated
DNA copy number and genotype alterations in cervical cancer cell lines in detail
using array comparative genomic hybridization (CGH) and single nucleotide
polymorphisms (SNP) array, as described in chapter 6. Furthermore, an effect of
copy number alteration on gene expression was investigated. Finally, in chapter 7
the results of the above mentioned studies are discussed and summarized.

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