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SYNDECAN-1 EXPRESSION AND SOLUBLE SYNDECAN-1 IN HEAD AND NECK AND LUNG CARCINOMAS

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Academic Dissertation

To be publicly discussed, with the permission of the Medical Faculty of the University of Helsinki, in the Auditorium of the Department of Oncology, Helsinki University Central Hospital, Haartmaninkatu 4, on January 13th, 2006, at 12 o'clock noon.

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To Maria, Mikko and Erika

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals:

I Anttonen A, Kajanti M, Heikkilä P, Jalkanen M, Joensuu H. Syndecan-1 expression has prognostic significance in head and neck carcinoma. British Journal of Cancer (1999) 79: 558–564.

II Anttonen A, Heikkilä P, Kajanti M, Jalkanen M, Joensuu H. High syndecan-1 expression is associated with favourable outcome in squamous cell lung carcinoma treated with radical surgery. Lung Cancer (2001) 32: 297–305.

III Joensuu H, Anttonen A, Eriksson M, Mäkitaro R, Alfthan H, Kinnula V, Leppä S. Soluble syndecan-1 and serum basic fibroblast growth factor are new prognostic factors in lung cancer. Cancer Research (2002) 62: 5210–5217.

IV Anttonen A, Leppä S, Ruotsalainen T, Alfthan H, Mattson K, Joensuu H. Pretreatment serum syndecan-1 levels and outcome in small cell lung cancer patients treated with platinum-based chemotherapy. Lung Cancer (2003) 41: 171–177.

V Anttonen A, Leppä S, Heikkilä P, Grenman R, Joensuu H. Effect of treatment of larynx and hypopharynx carcinomas on serum syndecan-1 concentrations. (submitted)

2. ABBREVIATIONS

bFGF = basic fibroblast growth factor
BSA = bovine serum albumin
CEA = carcinoembryonic antigen
EGF = epidermal growth factor
ELISA = enzyme-linked immunosorbent assay
FGFR = fibroblast growth factor receptor
FiRE = FGF-2 inducible response element
GAG = glycosaminoglycan
HGF = hepatocyte growth factor
HSPG = heparan sulphate proteoglycan
NSCLC = non-small cell lung cancer
PBS = phosphate-buffered saline
PDGF = platelet derived growth factor
PDZ = PSD-95, Dlg, ZO-1
PG = proteoglycan
PSA = prostate-spesific antigen
RT = room temperature
SCC = squamous cell carcinoma
SCLC = small cell lung cancer
TGF-β = transforming growth factor β

3. ABSTRACT

The syndecans are a family of transmembrane heparan sulphate proteoglycans (HSPGs) present on most cell types. With the lipid-linked glypicans, syndecans are the major source of heparan sulphates on the cell surface. Syndecan-1 is the most studied and best characterized member of the syndecan family. It mediates basic fibroblast growth factor (bFGF) binding and activity. Expression of syndecan-1 is down-regulated in many cell transformation models. The extracellular syndecan-1 domains can be shed from the cell surface in a highly regulated process called ectodomain shedding, giving rise to soluble syndecan-1 that can be measured from the serum.

Tissue expression of syndecan-1 is associated with a favourable outcome in several types of human squamous cell carcinomas (SCCs) when assessed with immunohistochemistry. We found low syndecan-1 expression to be associated with unfavourable overall survival in a univariate analysis in a series of 175 patients diagnosed with SCC of the head and neck (study I; P=0.001), in another series of 116 patients who had SCC of the lung (study II; P=0.026), and in a third series consisting of 44 patients with logoregional SCC of the larynx or the hypopharynx (study V; P=0.027). Syndecan-1 expression was generally higher in well differentiated head and neck and lung cancers than in moderately or poorly differentiated cancer (studies I and II). Syndecan-1 expression was an independent prognostic factor in a multivariate survival analysis that contained the histological grade as a covariate in SCC of the head and neck (P=0.016; relative risk, RR 1.9; 95% confidence interval, CI, 1.2–3.1, study I).

The influence of soluble serum syndecan-1 concentration on outcome was evaluated in a series of 134 patients diagnosed with small cell lung cancer (SCLC, studies III and IV), in another series of 138 patients diagnosed with non-small cell lung cancer (NSCLC, study III), and in a third series consisting of 44 patients with SCC of the head and neck (study V). A high serum syndecan-1 level turned out to be an independent prognostic factor both in NSCLC (P=0.011, RR 1.8, 95% CI 1.1–3.1, study III) and in SCLC (P=0.044, RR 1.68, 95% CI 1.02–2.77, study IV). A high serum bFGF level was associated with poor prognosis in NSCLC in a multivariate survival analysis (study III, P=0.056, RR 1.6, 95% CI 1.0–2.7), and in SCC of the head and neck in a univariate survival analysis (study V, P=0.043). Patients with head and neck cancer whose serum syndecan-1 level decreased more than 10% from the pretreatment level had a more favourable survival than those whose serum levels remained stable or increased (study V, P=0.0069). Recurrent head and neck cancer was found to be associated with elevated serum syndecan-1 levels as compared to the levels measured 3 months after the completion of the primary treatment (study V). This finding suggests that a part of serum soluble syndecan-1 originates from the tumour tissue.

4. INTRODUCTION

Heparan sulphate proteoglycans (HSPGs) are widely distributed in mammalian tissues and they participate in a variety of normal tissue functions. HSPGs are essential for normal tissue growth and development. Their expression is regulated during organ development and altered in various pathophysiological processes including cancer. The syndecans are a family of transmembrane HSPGs, which, together with the lipid-linked glypicans, are the major source of heparan sulphate on cell surfaces (reviewed by Bernfield et al., 1999).

The syndecan family consists of 4 members (syndecan-1, syndecan-2, syndecan-3, and syndecan-4) encoded by four different genes. Each member has its own tissue distribution (reviewed by Bernfield et al., 1992); syndecan-1 is expressed mostly by epithelial cells. Its expression is altered in malignant tumours and decreases as an *in situ* carcinoma progresses to an invasive carcinoma (reviewed by Sanderson, 2001, and by Sasisekharan et al., 2002). Basic fibroblast growth factor (bFGF) is a heparin-binding molecule that binds to syndecans and remains biologically active when immobilized in the cell matrix via the heparan sulphate chains of syndecan (Salmivirta et al., 1992a).

In clinical series syndecan-1 expression, as detected by immunohistochemistry, has been found to be associated with a poor histological grade of cancer differentiation in a few types of human cancer, and a low syndecan-1 expression in cancer tissue has been associated with poor outcome in head and neck cancer, mesothelioma, gastric cancer, cholangiocarcinoma, and NSCLC (Inki et al., 1994a; Pulkkinen et al., 1997; Kumar-Singh et al., 1998; Wiksten et al., 2000; Harada et al., 2003; Shah et al., 2004). The extracellular syndecan-1 domain can be shed from the cell surface into the extracellular fluid by ectodomain shedding (Kim et al., 1994; Fitzgerald et al., 2000). The serum syndecan-1 concentrations found in healthy individuals are, however, low (Seidel et al., 2000a). A high soluble serum syndecan-1 concentration has been found to have adverse prognostic value in multiple myeloma (Seidel et al., 2000a)

5. REVIEW OF THE LITERATURE

5.1. Cell surface heparan sulphate proteoglycans

Proteoglycans (PGs) are ubiquitous molecules that carry sulphated carbohydrate side chains called glycosaminoglycans (GAG). PGs consist of a diverse group of proteins that are grouped together mainly because they contain GAG. There are four main types of GAGs: heparin/heparan sulphate, chondroitin sulphate/dermatan sulphate, keratan sulphate, and hyaluronic acid (reviewed by Ruoslahti, 1988; and by Kjellen and Lindahl, 1991). HSPGs are composed of a core protein with at least one covalently bound GAG chain (reviewed by Kjellen and Lindahl, 1991). These chains contain regions with a high level of sulfation and epimerization, sequences with alternating N-sulfation and N-acetylation, and unmodified domains which are mostly N-acetylated and contain little sulphate (reviewed by Tumova et al, 2000).

According to their predominant cellular location, PGs can also be classified as cell surface PGs (e.g. syndecans, glypicans, betaglycan, CD44), extracellular matrix PGs (e.g. perlecan), and intracellular granule proteoglycans (e.g. serglycin, reviewed by Kjellen and Lindahl 1991). Two major groups of cell surface PGs are the transmembrane syndecans (reviewed by Bernfield et al., 1992; and by Carey, 1997) and glycosylphosphatidylinositol-anchored glypicans (David et al., 1990). CD44E, which is a splice variant of the matrix receptor CD44 with a single HS chain and betaglycan, can act as a ”part time” PG (Brown et al., 1991; Lopez-Casillas et al., 1991). These proteins may bear heparan sulphate chains in some proportion or under some conditions.

Cell surface HSPGs bind extracellular proteins and form signaling complexes with receptors. HSPGs immobilize proteins at the cell surface and mediate protein internalization. HSPGs have a role in cell adhesion and migration. These processes may involve intact PG and require both heparan sulphate-ligand-binding and direct or indirect interactions of the core protein with cytoskeletal and/or signaling molecules (reviewed by Tumova et al., 2000). The cell surface HSPGs bind to various ligands such as growth factors, cytokines, cells, extracellular matrix components and microbes (reviewed by Bernfield et al., 1999; and by Tumova et al., 2000).

The cell surface HSPGs can also act as coreceptors for soluble growth factors (reviewed by Sasisekharan et al., 1997; by Faham, 1998; by Gallagher, 2001; and by Mohammadi et al., 2005). Growth factors that bind to heparan sulphate or heparin include the family of FGFs (reviewed by Gallagher, 2001; and by Wu et al., 2003), hepatocyte growth factor

(HGF) (Lyon and Gallagher, 1994), platelet-derived growth factor (PDGF) (Feyzi et al., 1997), heparin-binding epidermal growth factor (EGF)-like growth factor (Higashiyama et al., 1993), and vascular endothelial growth factor (Neufeld et al., 1999). In addition, γ-interferon (Lortat-Jacob et al., 1995) and various chemokines (McFadden and Kelvin, 1997) bind to HSPGs. HSPGs and heparin are known to prevent blood coagulation by interfering with thrombin processing fibrinogen to fibrin (Conrad, 1998; Bourin and Lindahl, 1993) and to be involved in lipid metabolism (Fuki et al., 1997; Rosenberg et al., 1997). HSPGs also participate in the catabolism of proteins implicated in Alzheimer's disease (Donahue et al., 1999) and are implicated in the binding of various pathogens and mediating their invasion into host cells (reviewed by Rostand and Esko, 1997; Sawitzky, 1996). Some of the cell surface HSPGs (e.g. syndecans and glypicans) undergo regulated proteolytic cleavage near the plasma membrane and release their ectodomains as soluble intercellular regulators resulting in soluble PGs that probably retain all the binding propeties of their parent molecules (reviewed by Bernfield et al., 1999).

5.2. The syndecan family

Syndecans are type I transmembrane proteins that share distinctive structural features (reviewed by Bernfield et al., 1992; by David, 1993; and by Carey, 1997). They have diverse functions ranging from participation in cell-cell adhesion, regulation of cell signaling by growth factors, and organization of cell-matrix adhesions. There are four genes in the vertebrate syndecan family that each encode one type of syndecan, i.e. syndecan-1 ("syndecan", CD138), syndecan-2 (fibroglycan), syndecan-3 (N-syndecan) and syndecan-4 (ryudocan, amphiglycan).

The ectodomain of all syndecan core proteins has 3 to 5 putative GAG attachment sites near the N-terminus that are usually heparan sulphate substituted, although some members can be substituted with chondroitin or dermatan sulphate. In addition, syndecan-1 and -3 proteins have 2 or 3 putative membrane-proximal GAG attachment sites. The transmembrane domains of syndecans are highly homologous. Within the small cytoplasmic domains there are 2 regions of high sequence conservation (the conserved regions C1 and C2) split by an interventing sequence (V region) that is specific to individual syndecans (reviewed by Woods and Couchman, 1998, by Woods, 2001, and by Beauvais and Rapraeger, 2004). These highly conserved domains bind to a number of cytoplasmic and cytoskeletal proteins (reviewed by Zimmermann and David, 1999).

All syndecans have a COOH-terminal FYA sequence in C2 that can interact with the PDZ domains of several protein (post-synaptic density 95, PSD-95; discs large, Dlg; and zonula occludens-1, ZO-1 domains). The first PDZ protein shown to interact with syndecans was

syntenin (Asundi et al., 1995; Grootjans et al., 1997 & 2000; Zimmermann et al., 2001). Other PDZ domain proteins that bind the syndecans include CASK/Lin-2 (Cohen et al., 1998; Hsueh et al., 1998), synbindin (Ethell et al., 2000), and synectin (Gao et al., 2000). The C1 region interacts with the members of src/cortactin signaling pathway (syndecan-3, reviewed by Couchman et al., 2001, by Woods and Couchman, 2000 and by Tumova et al., 2000), ezrin (syndecan-2, Granes et al., 2000), and syndesmos (syndecan-4, Baciu et al., 2000). The V regions of syndecans are conserved between species but differ between syndecans 1 to 4 (Oh et al., 1998; reviewed by Beauvais and Rapraeger, 2004). Structure of syndecan-1 is shown in Figure 1.

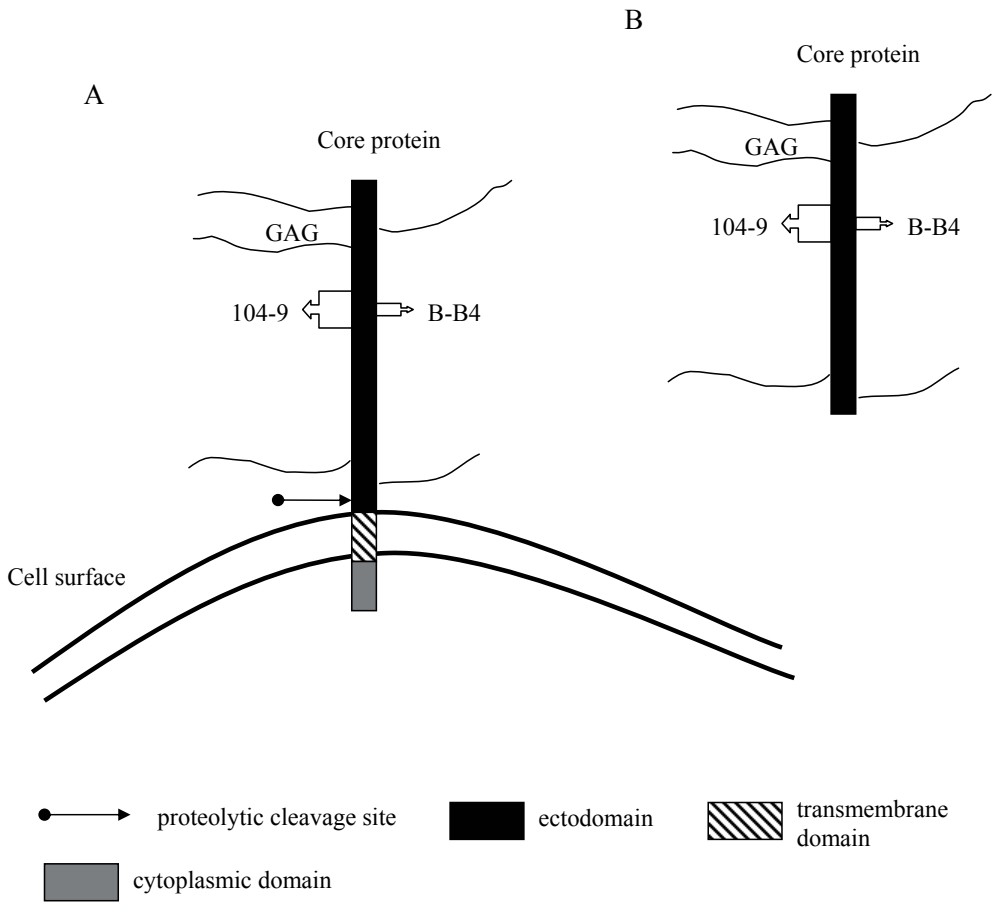

Figure 1. Schematised structure of syndecan-1 at the cell surface and the shed syndecan-1 ectodomain. A) The 310-amino-acid long core protein of human syndecan-1 consists of 3 domains: short cytoplasmic domain, transmembrane domain and extracellular domain (ectodomain). The ectodomain contains 5 possible GAG attachement sites, which are at positions 37, 45, 47, 206 and 216 (Mali et al., 1990). The epitope of B-B4 has been localized between amino acids 90–93 and the epitope of 104–9 between amino acids 88–110. B) Syndecan-1 can be cleaved from the cell surface near the plasma membrane and released into the extracellular space in a process known as shedding.

Virtually all cell types express at least one form of syndecan, but most express multiple syndecans. Syndecan expression is highly regulated and distinctive patterns of syndecan expression characterize individual cell types, tissues and development stages. In general, syndecan-1 is the major syndecan in epithelial cells, syndecan-2 in fibroblasts, syndecan-3 in neuronal tissues and interestingly, syndecan-4 is present in a range of cell types including fibroblasts, epithelial, and smooth muscle cells (reviewed by Bernfield et al., 1992, and by Woods, 2001).

5.3. Syndecan-1

5.3.1. A short history of syndecan-1

Syndecan-1 is the most studied member of the syndecan family. It was originally isolated from a mouse mammary epithelial cell line (Rapraeger and Bernfield, 1983), and was later identified as a developmentally regulated type-I-transmembrane protein that binds to the extracellular matrix components surrounding epithelial cells (Rapraeger and Bernfield, 1983; Koda and Bernfield, 1984; Saunders and Bernfield, 1988). The protein was named as syndecan from the Greek word *syndein* (to bind together) as it was thought to link the cytoskeleton to the interstitial matrix (Saunders et al., 1989). Syndecan-1 was found to be a hybrid PG, containing both heparan sulphate and chondroitin sulphate chains attached to the protein core (Rapraeger et al., 1985), but later it was found that syndecan-1 may also carry heparan sulphate alone (Sanderson et al., 1989; Salmivirta et al., 1991). Saunders et al. cloned and sequenced full-length syndecan-1 cDNA using a library of mammary epithelial cells, and assessed the expression of its mRNA in various tissues (1989). The 311-amino acid core protein was found to have a unique sequence that contained several structural features consistent with its role as a matrix anchor and as an acceptor of two distinct types of GAG chains. Human syndecan-1 gene was mapped to chromosome 2p23 (Ala-Kapee et al., 1990).

5.3.2. Tissue expression of syndecan-1

In the developing mouse syndecan-1 expression is detected initially at the 4-cell stage (Sutherland et al., 1991). After gastrulation, highest expression is found in the ectoderm and in the endoderm that differentiate into syndecan-1 positive epithelial stuctures found in adults (Hayashi et al., 1987; Sutherland et al., 1991). Syndecan-1 expression is found in various developing epithelia such as the epidermis, and the mammary epithelium (Trautman et al., 1991). During embryonic development syndecan-1 is also expressed in the mesenchyme at sites of epithelial-mesenchymal interactions e.g. in the developing teeth (Thesleff et al.,

1988; Vainio et al., 1991), the kidney (Vainio et al., 1992), the lung (Brauker et al., 1991), the hair follicles (Trautman et al., 1991), and the limb (Solursh et al., 1990).

In a normal adult syndecan-1 is abundantly expressed on epithelial tissues, where the strongest expression is found in the stratified epithelia at the cell-cell contacts between epidermal keratinocytes (Hayashi et al., 1987; Inki et al., 1991). In keratinocytes, syndecan-1 is localized at the cell surface, predominantly in the suprabasal cell layers, whereas the basal cell layer shows only weak expression. Fibroblasts (Elenius et al., 1992) and endothelial cells (Elenius et al., 1991; Kojima et al., 1992) express small amounts of syndecan-1. Syndecan-1 is also present on pre-B cells (Sanderson et al., 1989), plasma cells (Hayashi et al., 1987), myeloma cells (Ridley et al., 1993; Wijdenes et al., 1996), and Leydig cells (Hayashi et al., 1987). Expression of syndecan-1 on B-cells is regulated by interleukin-6 (Sneed et al., 1994; Götte et al., 2002; Götte, 2003), and it is lost in pre-B-cells immediately before the mature B-cells are released into the circulation (Sanderson et al., 1989).

In the process of wound healing syndecan-1 is transiently decreased in keratinocytes migrating into the wound, whereas it is increased in the keratinocytes proliferating at the wound margin and in endothelial cells within the granulation tissue, while syndecan-4 is induced on the fibroblasts that form granulation tissue (Elenius et al., 1991; Gallo et al., 1996). A reduction of syndecan-1 immunostaining has been found in the reparative epithelium in inflammatory bowel disease (Day et al., 1999a), and the loss of syndecan-1 may lead to impaired binding of bFGF and a reduced rate of ulcer healing (Day and Forbes, 1999b). Tanabe and coworkers (1999) studied syndecan-1 expression in gastric mucosal ulcers, and found enhanced expression in elongated regenerative foveolar cells at the healing ulcer margins and within the early scar tissues. Balloon catheter induced vascular wall injury increases syndecan-1 expression in carotic artery smooth muscle cells (Cizmeci-Smith et al., 1997a; Wang et al., 1997).

Expression of syndecan-1 decreases in cellular transformation models. When syndecan-1 was transfected into S115 mouse mammary tumour cells, the re-expression of syndecan-1 restored the epithelial morphology of the S115 cells (Leppä et al., 1992). Conversely inhibition of syndecan-1 expression in NMuMG cells caused them to become mesenchyme-like (Kato et al., 1995). Liu and coworkers (1997) irradiated the mouse skin, which resulted in loss of syndecan-1 expression. Syndecan-1 expression is downregulated or lost in a number of epithelial cancers, and in premalignant lesions of the uterine cervix and the oral mucosa. Such a loss may be an early genetic event contributing to tumour progression (Inki et al., 1992, Inki et al., 1994b & 1994c; Hirabayashi et al., 1998; Nakanishi et al., 1999; Rintala et al., 1999; Soukka et al., 2000; Sanderson, 2001; Numa et al., 2002). In premalignant areas of stratified epithelia syndecan-1 is lost from the basally located, atypical cell layers (Inki et al., 1991).

5.3.3. Regulation of syndecan-1 expression

Syndecan-1, like other members of the syndecan family, is expressed in a distinct cell-, tissue-, and development-specific pattern (Kim et al., 1994). Expression of syndecan-1 is also modified in pathological conditions. The molecular mechanisms that are responsible for the regulation of syndecan-1 expression are under active investigation.

Expression of syndecan-1 can be induced in cultured cells by treatment with various growth factors and cytokines. bFGF alone (Jaakkola et al., 1997) or in a combination with transforming growth factor β (TGF-β) increased syndecan-1 expression in 3T3 cells (Elenius et al., 1992). In vascular smooth muscle cells PDGF or angiotensin II stimulated syndecan-1 expression (Cizmeci-Smith et al., 1993). PDGF and TGF-β increased syndecan-1 expression in human periodontal fibroblasts and osteoblasts, while IL-1b decreased the expression (Worapamorn et al., 2002). Gallo and coworkers (1994) showed induced syndecan-1 expression in cultured mouse mesenchymal cells during wound repair using a proline-rich antimicrobial peptide (PR-39) that is released by inflammatory cells entering the skin. In endothelial cells, in turn, syndecan-1 expression was decreased by tumour necrosis factor-α (Kainulainen et al., 1996).

In general, levels of syndecan-1 synthesis correlate with syndecan mRNA levels, which suggests that its regulation is mainly controlled at transcriptional level. The upstream sequences of the syndecan-1 gene have promoter activity and contain TATA and CAAT boxes as well as a variety of other potential binding sites for transcription factors, including Sp1, NF-kappa B, MyoD, and Antennapedia (Hinkes et al., 1993; Vihinen et al., 1996). Syndecan-1 expression is down-regulated during myogenesis. It is modulated by bFGF and TGF-β, which when added together induced syndecan-1 expression. Retinoic acid, an inducer of myogenesis, inhibited syndecan-1 expression and abolished the effect of the growth factors via a proximal promoter region (Larrain et al., 1997). The DNA-binding protein encoded by Wilms' tumour suppressor gene *wt* 1 has been found to function as a transcriptional activator of syndecan-1 expression (Cook et al., 1997). A far upstream enhancer FGF-2 inducible response element (FiRE) on the syndecan-1 gene was shown to mediate syndecan-1 induction by FGFs in fibroblasts and by EGF in keratinocytes, but not *vice versa* (Jaakkola et al., 1997; Jaakkola et al.,1998a). During wound healing the activation of FiRE is seen in the migrating keratinocytes and might be responsible for the induced syndecan-1 expression in these cells (Jaakkola et al., 1998b); syndecan-1 expression is increased up to 20-fold in keratinocytes during wound healing (Elenius et al., 1991). Syndecan-1 induction during wound healing may be enhanced by syndecan-inducing antimicrobial peptides released into the wound from inflammatory cells (Gallo et al., 1996).

Syndecan-1 expression may also be regulated post-transcriptionally. Syndecan-1 expression was strongly induced in stratifying keratinocytes (Sanderson et al., 1992a) and in mesenchymal cells condensing during kidney formation (Vainio et al., 1989a), but no simultaneous change in the mRNA levels was found. Similarly, thioglycollate-elicited peritoneal mouse macrophages contain 9-fold more syndecan-1 mRNA than untreated macrophages, but this mRNA is not translated into the syndecan-1 core protein unless the cells are treated with agents that raise the intracellular cAMP levels (Yeaman and Rapraeger, 1993). Post-translationally regulated syndecan-1 expression also occurs (Sanderson and Bernfield, 1988). Shedding of the extracellular domain of syndecan-1 from the cell surface provides an additional potential level for functional control (Jalkanen et al., 1987).

5.3.4. Shedding of syndecan-1 and soluble syndecan-1

A small proportion of membrane-anchored proteins undergo regulated endogenous proteolytic cleavage resulting in release of their ectodomains as soluble intercellular regulators in the process of ectodomain shedding (Hooper et al., 1997; Werb and Yan, 1998). Shedding liberates these ectodomains as soluble molecules and at the same time their concentration at the cell surface decreases. The released fragments retain the ability to bind the same extracellular ligands as the heparan sulphate chains at the cell surface, and they can compete for the same ligands as their cell surface counterparts. Since syndecan-1 shedding is regulated in normal cells by physiological mediators, shedding is likely a response to specific developmental and pathophysiological cues (Fitzgerald et al., 2000).

The syndecan-1 core protein is cleaved on the cell surface at the extracellular juxtamembrane site (Figure 1), and the proteolytic activity responsible for accelerated shedding differs from that involved in constitutive shedding of syndecan ectodomains (Fitzgerald et al., 2000). The intact ectodomains of each mammalian syndecan and the *Drosophila* syndecan are constitutively shed into the conditioned media of cultured cells (Kim et al., 1994; Spring et al., 1994) as part of normal cell surface turnover (Yanagishita and Hascall, 1992; Yamagata et al, 1993; Yanagishita, 1998), but the syndecan-1 concentrations found in sera of healthy individuals are low (Seidel et al., 2000a). Shed syndecan-1 ectodomains are found in fluids that accumulate following tissue injury and inflammation (Subramarian et al., 1997, Kato et al., 1998). Presence of excessive amounts of soluble syndecan-1 ectodomain in a wound modulates the function of proteases and inhibits growth factor action, which may delay skin wound repair (Elenius et al., 2004). High levels of soluble syndecan-1 are also found in sera and bone marrow aspirates of patients with multiple myeloma (Seidel et al., 2000a). Of note, *in vivo* soluble syndecan-1 promotes growth of myeloma tumours (Yang et al., 2002).

Shedding is a highly regulated process and can be induced by various agents, of which the phorbol esters are the best characterized agonists (Hooper at al., 1997; Subramanian et al., 1997). Shedding of syndecan-1 can be enhanced by direct proteolytic cleavage (e.g. by thrombin, plasmin, elastase or matrix metalloproteinases; Subramanian et al., 1997; Buczek-Thomas and Nugent, 1999; Endo et al., 2003), and by cellular stress (mechanical, heat shock, or hyperosmolarity) involving stress-activated protein kinase pathways (Fitzgerald et al., 2000), and by growth factors (Subramanian et al., 1997; Kato et al., 1998). Ectodomain shedding is inhibited by the tissue inhibitor metalloproteinase 3 (TIMP), but not by TIMP- 1 or -2 (Fitzgerald et al., 2000).

5.3.5. Functions of syndecan-1 and its association with bFGF

Earlier studies in cell culture conditions have shown that syndecan-1 binds various matrix proteins via its heparan sulphate chains, e.g. type I, III, and V collagens (Koda et al., 1985), fibronectin (Saunders and Bernfield, 1988), thrombospondin (Sun et al., 1989), tenascin (Salmivirta et al., 1991), amphoterin (Salmivirta et al., 1992b), and laminin (Salmivirta et al., 1994). Syndecan-1 polarizes to the basolateral surfaces of cultured epithelial cells (Rapraeger et al., 1986) and of simple epithelia (Hayashi et al., 1987), and localizes during early embryogenesis to the intial sites of extracellular matrix accumulation (Sutherland at al., 1991). During tooth development syndecan-1 colocalizes with tenascin (Vainio et al., 1989b). It is expressed on the developing B-cells only when they are in contact with the extracellular matrix (Sanderson et al., 1989), and later it binds B-cells to type I collagen (Sanderson et al., 1992b).

Syndecan-1 has potential ability to regulate cytoskeletal organization and to associate with the actin cytoskeleton (reviewed by Carey, 1997; and by Yoneda and Couchman, 2003). For example, syndecan-1-expressing Raji lymphoid cells (a human lymphoblastoid cell line) bind avidly and spread rapidly when they attach to extracellular matrix ligands that contain heparan sulphate-binding domains (Lebakken et al., 2000; McQuade and Rapraeger, 2003). Transfection of syndecan-1 into COS-7 cells stimulates cell migration (Adams et al., 2001). Ectopic expression of syndecan-1 in Schwann cells, which normally do not express syndecan-1, results in enhanced migration of the cells on fibronectin and laminin-coated substrata, and in a striking reorganization of Schwann cell microfilaments (Carey et al., 1994).

Syndecans have been suggested to play a role in cell-cell adhesion based on the finding that several well-characterized cell-adhesion molecules bind to heparan sulphate (reviewed by Carey, 1997). Certain myeloma cell lines have lost the ability to synthesize syndecan-1, and the loss of syndecan-1 expression correlates with their inability to adhere to one another

(Sanderson et al., 1992b). Stable transfection of these cells with syndecan-1 cDNA restores the cell-to-cell adhesion activity (Stanley et al., 1995). In contrast, Raji cells do not undergo cell-cell adhesion following their transfection with syndecan-1 (Rapraeger, 1993).

Syndecan-1 expression is needed for maintenance of a differentiated epithelial phenotype. It affects the organization of the actin cytoskeleton and the expression of E-cadherin, which is reponsible for epithelial intercellular adhesions (Kato et al., 1995). When endogenous syndecan-1 expression is suppressed by transfection of antisense cDNA, the cell morphology changes markedly (Kato et al., 1995). This dramatic change is accompanied by a loss of E-cadherin expression. In malignant tumour cells where E-cadherin expression was downregulated by using specific antisense RNAs, syndecan-1 expression was suppressed, and transfection of E-cadherin cDNA resulted in the upregulation of syndecan-1 expression in association with an epithelial phenotype and decreased invasiveness (Leppä et al., 1996). Epithelial cells that have lost syndecan-1 expression grow readily in soft agar, indicating that they have lost their characteristic anchorage-dependent growth (Kato et al., 1995), and addition of purified syndecan-1 ectodomain inhibites the growth of carcinoma cells *in vitro* (Mali et al., 1994).

Cell surface syndecan-1 inhibits invasion of tumour cells into 3-dimensional gel composed of type I collagen. This inhibition is dependent on the syndecan-1 heparan sulphate chains, although a role for the syndecan-1 ectodomain core protein has also been shown. The anti-invasive effect of syndecan-1 is dramatically reduced by deletion of an ectodomain region located close to the plasma membrane, and that this domain is functionally specific, because its deletion did not affect syndecan-1-mediated cell binding to collagen or cell migration (Langford et al., 2005).

Syndecan-1 functions have also been studied *in vivo*, which support *in vitro* studies. Studies in syndecan-1-knockout mice revealed an essential role for syndecan-1 in mediating cell proliferation and in regulation of integrin expression during wound healing (Stepp et al., 2002). Healing problems in these mice were thought to result primarily from defects in fibroblast migration, but Stepp et al. showed in 2002 that the delayed healing resulted from impaired keratinocyte function. This suggests that syndecan-1 has a role as a regulator of gene transcription in keratinocytes and in keratinocyte activation after injury (Stepp et al., 2002). In contrast, the role of syndecan-1 in tumour formation appears to be more complex as syndecan-1 does not prevent but supplements Wnt-1-induced mammary tumourigenesis in mice (Alexander at al., 2000) and promotes formation of metastases in a mouse lung SCC model (Hirabayashi et al., 1998).

Many growth factors bind to heparin or heparan sulphate, including the FGF family members (Nugent and Iozzo, 2000; Ornitz and Itoh, 2001). Over 20 mammalian FGFs have been characterized to date, but bFGF remains the most studied member of the family. Members of each of the FGF subclass are capable of binding to one or more of 4 different FGF receptors (FGFR1-4). Binding of FGFs to heparan sulphate is needed for signal transmission through the FGF high-affinity receptors (Rapraeger et al., 1991; Yayon et al., 1991). It has been challenging to determine the role of syndecan-1 in bFGF receptor signaling. It was first found that enhanced syndecan-1 expression in cultured cells strongly inhibited bFGF-induced cell proliferation (Mali et al., 1993). Later, purified preparations of soluble syndecan-1 were found to inhibit bFGF-receptor binding in cell free assays (Avierez et al., 1994). Finally, syndecan-1 expression was shown to stimulate FGFR-1 signaling (Steinfeld et al., 1996) and to mediate bFGF binding and activity (Filla et al., 1998). A strong influence of syndecan-1 expression on cell responsiveness to bFGF has been shown in *in vitro* models, where syndecan-1 expression has resulted in a several-fold increase in cell sensitivity to bFGF (Larrain et al., 1998). Syndecans have been implicated as modulators of the bFGF receptor-binding affinity, giving rise to decreased bFGF binding as a response to increased cell density in a culture (Richardson et al., 1999). Expression of syndecan-1 core protein in breast carcinoma tissue correlates well with the bFGF receptor complex formation *in situ*, suggesting that in breast carcinomas bFGF receptor binding is influenced by the syndecan-1 core protein levels (Mundhenke et al., 2002). Soluble syndecan-1 ectodomains found in inflammatory fluids appear to act as inhibitors of bFGF activity (Kato et al., 1998).

Syndecan-1 modulates hepatocyte growth factor (HGF) activity in myeloma. Purified soluble syndecan-1 can displace HGF from myeloma cell surface suggesting that soluble syndecan-1 may act as a carrier of HGF *in vivo* (Seidel et al., 2000b). Soluble syndecan-1 promotes growth of myeloma tumours *in vivo*, and both in *in vitro* and *in vivo* studies the syndecan-1 ectodomain promotes dissemination of myeloma cells (Yang et al., 2002).

Syndecan-1 also binds extracellular ligands that are neither growth factors nor cell adhesion molecules. These include lipoprotein metabolism proteins. Syndecan-1 may mediate LDL receptor-independent clearance of lipoproteins via binding to lipoprotein lipase, an enzyme involved in triglyceride catabolism (Fuki et al., 1997). Another class of enzymes that may be regulated by heparin/heparan sulphate is certain serine proteases and their inhibitors (serpins). Studies on the binding activity of antithrombin III (AT III), a serpin that inhibits thrombin and other coagulation proteases, revealed that thrombin increased synthesis of syndecan-1 that in turn exhibited high-affinity AT III binding (Cizmeci-Smith and Carey, 1997b). Syndecan-1 has also been found to mediate the invasion of a host by pathogenic cells such as *Neisseria gonorrhoeae* (Freissler et al., 2000).

It has been suggested that syndecan-1 expression may prevent bone destruction, since syndecan-1 has been found to increase osteoblast development and inhibit osteoclast formation (Dhodapkar et al., 1998).

5.3.6. Clinical significance of syndecan-1

Based on the studies carried out *in vitro* and in experimental models, it can be anticipated that syndecan-1 expression may influence the clinical behaviour of human cancer. HSPGs regulate several aspects of cancer progression including tumorigenesis and metastasis (reviewed by Blackhall et al., 2001; and by Sasisekharan et al., 2002), and in squamous cell carcinomas syndecan-1 expression appears to be associated with maintenance of the differentiated morphology of cancer and inhibition of cancer invasiveness. Expression of syndecan-1 is sometimes downregulated in tumours, in premalignant lesions, and as an *in situ* carcinoma processes to an invasive carcinoma (Inki and Jalkanen, 1996; Nakanishi et al., 1999; Rintala et al., 1999; Soukka et al., 2000; Sanderson , 2001). However, in some experimental models syndecan-1 has promoted tumorigenesis and formation of metastases (Hirabayashi et al., 1998; Alexander et al., 2000).

5.3.6.1. Lung cancer

Several human lung cancer cell lines express syndecan-1 (Nackaerts et al., 1997; Toyoshima et al., 2001; Nanki et al., 2001, Matsunaga et al., 2002). Nackaerts and coworkers found all 3 squamous cell lung cancer cell lines tested to be positive for syndecan-1, and 2 of the 3 adenocarcinoma cell lines investigated also showed marked expression. Similar results were reported by Toyoshima and coworkers, who detected syndecan-1 mRNA in both NSCLC and SCLC cell lines. In the study carried out by Nanki and coworkers (2001), syndecan-1 expression was stronger in NSCLC than in SCLC cell lines. In mouse cell lines derived from SCC, increased syndecan-1 expression was associated with the rate of cell proliferation and it was reported to promote formation of metastases (Hirabayashi et al., 1998). Syndecan-1 gene transcripts were present in lung cancer tissue and in the peripheral blood of lung cancer patients, which might support the authors' hypothesis that isolation of syndecan-1 mRNA from the blood may aid in early detection and staging of lung cancer, and follow-up of lung cancer patients (Matsunaga et al., 2002).

Lung tumours have been found to express syndecan-1. In general, NSCLCs stain more often positively for syndecan-1 than SCLCs, SCCs of the lung express more often syndecan-1 than adenocarcinomas of the lung, and poorly differentiated tumours express markedly less syndecan-1 than better differentiated ones (Nackaerts et al., 1997; Toyoshima et al., 2001). Linnerth et al. (2005) found that syndecan-1 levels were elevated both in murine and human lung adenocarcinomas and SCCs. Importantly, the high syndecan-1 levels could already be

detected in small and node-negative lung tumours suggesting that syndecan-1 could serve as an early marker for lung tumour development. In line with the studies presented in this theses, one recent study comprising a series of 78 NSCLC patients found tumour tissue syndecan-1 expression to be associated with favourable survival, and tumour syndecan-1 expression turned out to be an independent prognostic factor in a multivariate survival analysis (Shah et al., 2004).

Syndecan-1 is strongly expressed in the epithelial type of mesothelioma and patients with strong syndecan-1 positive tumours have longer survival than those whose tumours show weak or negative staining for syndecan-1 (Kumar-Singh et al., 1998). Syndecan-1 expression has been studied as a marker for distinguishing adenocarcinoma from epitheloid mesothelioma. Adenocarcinoma cells were found to produce more mRNA for syndecan-1, whereas cells derived from the mesothelium expressed WT1, biglycan, and larger amounts of syndecan-2 (Dobra et al., 2000; Gulyas and Hjerpe, 2003).

5.3.6.2. Head and neck carcinoma

Syndecan-1 expression is enhanced during normal differentiation of cultured human keratinocytes derived from the oral mucosa, while malignant transformation of keratinocytes is associated with suppression of syndecan-1 expression (Inki et al., 1994b). In premalignant lesions, such as dysplastic oral epithelium, syndecan-1 expression is downregulated compared to normal oral squamous cell epithelium (Soukka et al., 2000). When paraffin-embedded or frozen tissues of SCC of the head and neck is used as the starting material, strong immunoreactivity for syndecan-1 has been found to be associated with a higher histological grade of differentiation (Inki et al., 1994a; Pulkkinen et al., 1997; Mikami et al., 2001; Klatka, 2002); strong syndecan-1 expression is more common in well or moderately differentiated carcinomas than in poorly differentiated ones.

There are few data available on the prognostic value of syndecan-1 expression in SCC of the head and neck. In the first study reported that addressed the question of syndecan-1 as a prognostic factor in invasive SCCs of the head and neck, Inki and her coworkers (1994a) found that patients with a syndecan-1-positive tumour had higher overall and recurrence-free survival rates than patients who had tumours showing weak or negative staining for syndecan-1 in a small series consisting of 29 patients. Similar results were later found in 2 studies that involved 100 and 48 patients with SCC of the larynx (Pulkkinen et al., 1997; Klatka, 2002). There are no data available on the prognostic value of soluble syndecan-1 in SCC of the head and neck.

5.3.6.3. Other malignancies

Several studies have addressed the role of syndecan-1 expression in other malignancies than cancers of the lung or the head and neck. The most studied single tumour type is B-cell lymphoid malignancies (reviewed by Sanderson and Borset, 2002). Syndecan-1 was detected on human myeloma cells by Western blotting in 1993 (Ridley et al., 1993), and it was subsequently identified on the myeloma cell surface by flow cytometry using a monoclonal antibody (Wijdenes et al., 1996). Syndecan-1 can also be found on the myeloma cells that circulate in the blood (Witzig et al., 1998; Bayer-Garner et al., 2001). Syndecan-1 has been considered to be a reliable and sensitive marker for plasmocytoma independent of cytological differentiation (Bayer-Garner et al., 2003). Syndecan-1 is expressed by many lymphomas. It is expressed on the Reed-Sternberg cells from patients with classic Hodgkin's disease, but not on the Reed-Sternberg cells present in the nodular lymphocyte predominant Hodgkin's disease (Carbone et al., 1997). It is currently somewhat controversial whether syndecan-1 is expressed on malignant cells of B-cell chronic lymphocytic leukemia (B-CLL). Syndecan-1 has been detected by polymerase chain reaction and immunostaining in the majority of B-CLL cases examined (Sebestyen et al., 1997; Sebestyen et al., 1999; Sutcliffe et al., 2000), but one study found only 1 of the 39 B-CLL cases examined to stain positively for syndecan-1 (Witzig et al., 1998).

Most myeloma cells in culture shed syndecan-1 ectodomain, which in turn induces apoptosis and inhibits the growth of the myeloma cells (Dhodapkar et al., 1998). In myeloma patients the levels of serum syndecan-1 ectodomain are elevated as compared to controls, and high serum syndecan-1 concentrations are associated with higher levels of marrow plasmocytosis, high serum β_2-microglobulin and paraprotein levels, and a large tumour mass (Dhodapkar et al., 1997). High serum levels of syndecan-1 have been reported to be a strong indicator of poor outcome and an independent prognostic marker in multiple myeloma (Seidel et al., 2000a; Aref et al., 2003; Kyrtsonis et al., 2004).

Studies based on immunohistochemistry and targeting on several types of human cancers including carcinoma of the breast (Stanley et al., 1999), uterine cervix (Inki et al., 1994c; Rintala et al., 1999), and colon and rectum (Day et al., 1999c), and basal cell and squamous cell skin carcinomas (Bayer-Garner et al., 1999; Bayer-Garner et al., 2000; Bayer-Garner et al., 2001; Mukunyadzi et al., 2002) suggest that syndecan-1 expression is dimished in malignant lesions as compared to premalignant and benign ones. In infiltrating ductal breast carcinoma staining for syndecan-1 is decreased as compared with the ductal epithelium of the normal breast and stromal-epithelial breast neoplasms. Unexpectedly, in carcinoma samples strong staining for syndecan-1 is present both within the connective tissue and on stromal cell surfaces, whereas syndecan-1 expression is absent in the stroma of the normal

breast and stromal-epithelial neoplasms (Stanley et al., 1999). Recently, two studies have concluded tumour syndecan-1 expression to be associated with a poor outcome in breast cancer (Barbareschi et al., 2003; Leivonen et al., 2004).

In primary invasive cervical carcinoma syndecan-1 expression was found to be associated with the histological differentiation grade and presence of squamous histology, but not with the clinical outcome (Rintala et al., 1999). In hepatocellular and colorectal carcinoma loss of syndecan-1 expression was associated with a low grade of differentiatiation and presence of metastases (Levy et al., 1996; Matsumoto et al., 1997; Fujiya et al., 2001). In contrast to the previous tumour types, in pancreatic cancer tissues syndecan-1 expression was found to be present at moderate to high levels in the majority of the cancer cells located within the tumour mass or in the metastatic lesions, while the expression remained low or moderate in the samples containing histologically normal pancreas and chronic pancreatis (Conejo et al., 2000). Similar results were obtained when Zellweger and coworkers (2005) evaluated the expression of syndecan-1 in advanced prostate carcinoma. They found marked syndecan-1 overexpression in hormone-refractory prostate cancer and metastatic tissue compared to localized prostate cancer. Among 551 patients with prostate carcinoma syndecan-1 overexpression was found to predict early recurrence and to be associated with poor tumour-specific survival, a high Gleason grade, high Ki-67 expression, and Bcl-2 overexpression (Zellweger et al., 2003).

Syndecan-1 expression has also been studied by immunohistochemistry in soft tissue tumours, but no correlation with the phenotype was found (Orosz and Kopper, 2001).

High syndecan-1 expression has been found to correlate with a favourable outcome in patients with gastric cancer in a univariate survival analysis, especially in the subgroup of patients with stage I disease (Wiksten et al., 2000). Interestingly, patients with positive syndecan-1 expression in extracellular matrix had a worse outcome than patients with syndecan-1-negative stroma (Wiksten et al., 2001). Unlike the normal squamous cell epithelium, the normal ovary does not express syndecan-1, and tumour stroma syndecan-1 expression was reported to be associated with poor survival in ovarian adenocarcinoma (Davies et al., 2004).

6. AIMS OF THE STUDY

The aims of the present thesis were:

- To investigate the expression and prognostic significance of syndecan-1 in head and neck cancer and in lung cancer (I,II,III,V)
- To investigate the presence and prognostic significance of serum syndecan-1 and bFGF in patients with lung cancer or head and neck cancer (III,IV,V)
- To study the effect of treatment on serum syndecan-1 levels (V)

7. MATERIALS AND METHODS

7.1. Patients, tissue and serum samples

Study I. We collected the clinicopathological data of 265 patients with histologically diagnosed SCC of the head and neck. Ninety patients were excluded because of insufficient data or non-available paraffin-embedded tissue leaving 175 patients in the analysis. The patients were treated with surgery and post-operative split-course irradiation in Helsinki University Central Hospital in the period 1975 to 1990. The minimum follow-up time of the patients after the diagnosis was 2 years. The prognostic significance of syndecan-1 expression was studied using paraffin-embedded tissue samples (TABLE 1).

Study II. The series consisted of 116 patients with operable primary NSCLC squamous cell histological type treated with radical surgery in the Helsinki University Central Hospital in the period 1982 to 1992. Clinical data were collected and paraffin-embedded tumour samples stored for analysis. Expression of syndecan-1 was studied using the paraffin-embedded tissue samples (TABLE 1). The minimum follow-up time of the patients still alive was 12 months.

Study III. The series consisted of 207 patients with lung cancer who had had a serum sample collected and stored before cancer treatment. The patients were treated at the Department of Internal Medicine, Oulu University Hospital, in the period 1990 to 1992. We excluded cases where no diagnostic biopsy sample was available (n=16) and those with histologically unclassified lung cancer (n=7), which left 184 patients in the analysis. Forty-six patients had SCLC, and the rest of the patients had NSCLC (adenocarcinoma, n=44; SCC, n=82; large cell carcinoma, n=9; adenosquamous cell carcinoma; n=3). The median follow-up time of the patients still alive was 8.1 years. Syndecan-1 expression was studied in formalin-fixed paraffin-embedded tumour tissue (TABLE 1). The prognostic significance of serum syndecan-1 and serum bFGF was studied from the sera taken prior to the treatment (TABLE 2). The serum samples had been stored at –20^{o}C.

Study IV. The series consisted of 88 patients with SCLC who were treated at the Department of Internal Medicine, Helsinki University Central Hospital, in 1990 to 1999. The patients participated in 2 randomized clinical trials, of which one was a multicenter trial to assess the therapeutic value of interferon in the treatment of SCLC (Ruotsalainen et al., 1999; Ruotsalainen et al., 2000). The total number of patients in these trials was 304, and those study participants who had a frozen pretreatment serum sample available and who had been treated at the Helsinki University Central Hospital entered the present study.

Serum syndecan-1 concentrations were determined from sera taken prior to initiation of chemotherapy (TABLE 2). The sera had been stored at –70°C. Serum samples taken both prior to and after the treatment were available in 7 patients (TABLE 2).

Study V. The study includes 54 patients with SCC of the head and neck who had serum samples collected before, during, and after treatment, and who were treated at the Turku University Central Hospital in 1997 to 2000. Ten patients were excluded because of insufficient clinicopathological data. The series consisted of 42 patients with laryngeal carcinoma and 2 with hypopharynx carcinoma. Thirty-four patients received radiotherapy; 15 were treated with preoperative hyperfractionated radiotherapy (1.6 Gy twice a day, 5 days a week, to a total dose of 62 to 68 Gy) followed by surgery. Nineteen patients received radiotherapy to the primary site to a total dose of 70 Gy given in 35 fractions, and when needed the neck was irradiated to a total dose of 50 Gy in 25 fractions excluding the spinal cord after 40 Gy. Nine of the patients had surgery only (7 had laser cordectomy, 1 total laryngectomia, 1 hemilaryngectomia). One patient died before any cancer treatment was given. The minimum follow-up time was 13 months for the patients still alive. Syndecan-1 expression was studied in 27 available paraffin embedded tumour samples (TABLE 1). Serum syndecan-1 and bFGF levels were measured from the samples taken before any cancer treatment took place, and from samples collected 3 months after initiation of cancer treatment. Serum syndecan-1 levels were also measured at the beginning and at the end of a radiotherapy course, at the time of disease progression, and after a second cancer treatment whenever a serum sample was available (TABLE 2).

TABLE 1. Tissue samples examined

Tumour type	N	Study
Squamous cell carcinoma of the head and neck • Larynx • Tongue • Oral cavity • Tonsil • Hypopharynx	175 51 50 44 15 15	I
Squamous cell lung cancer	116	II
Lung cancer • Squamous cell carcinoma • Adenocarcinoma • Small cell lung cancer	45 36 6 3	III
Squamous cell carcinoma of the head and neck • Larynx (vocal cord carcinoma excluded) • Vocal cord	27 18 9	V

TABLE 2. Serum samples analysed for syndecan-1 concentration

Tumour type	N	Study
Lung cancer		III
NSCLC	138	
SCLC	46	
Controls	100	
Small cell lung cancer, prior to therapy	88	IV
Small cell lung cancer, following therapy	7	
Controls	110	
SCC of the head and neck		V
Pretreatment sera	44	
Beginning of radiotherapy	44	
End of radiotherapy	10	
Post-treatment sera	41	
At disease progression	9	
After second cancer therapy	4	

7.2. Control subjects (studies III and IV)

Serum syndecan-1 levels were measured in 100 (III) and in 110 (IV) male subjects who took part in a randomised Finnish population-based prostate cancer screening trial in 1996-1998 (Määttänen et al., 2001). Prostate-specific antigen (PSA) was first measured from the sera, and syndecan-1 was assessed from the frozen left-over sera stored for one to 2 months. The median age of the controls was 64 (range, 58 to 71).

7.3. Immunohistochemical staining of tissue samples (studies I, II, III, and V)

Syndecan-1 expression was studied by immunohistochemistry using a mouse monoclonal antibody B-B4 generated against extracellular domain of human syndecan-1 (Serotec, Oxford, UK; I-III, V) (Wijdenes et al., 1996) or a rat monoclonal antibody 104–9 (I, II, Figure 1) directed against human syndecan-1 core protein aminoacid residues 88 to 110 (Pulkkinen et al., 1997; Gattei et al., 1999). The epitope of B-B4 has been localized between the amino acids 90 and 93 (Dore et al., 1998). Five μm paraffin sections were first mounted on Vectabond-coated slides. The slides were kept at +37° °C for 24 hours, and after that deparaffinised and dehydrated. They were then incubated with 2% normal goat serum in 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) for 20 minutes at room tempera-

ture (RT) followed by B-B4 or 104–9 antibody in 0.3% BSA overnight at RT at a concentration of 1:200 (B-B4, I–III), 1:150 (B-B4, V) or 1:100 (104–9). After washing twice with phosphate-buffered saline (PBS), the slides were incubated with Vectastain biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) when B-B4 was used, and with biotinylated anti-rat IgG (Vector Laboratories) when 104–9 was used in 0.3% BSA for 30 minutes at RT. After washing twice with PBS, avidin DH-biotinylated horseradish peroxidase mixture was applied according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA) for one hour at RT. After incubation, the slides were washed twice with PBS. For colour reaction the slides were incubated with 0.02% 3-amino-9-ethylcarbazole (in N,N-dimethylformamide) and 0.1% hydrogen peroxidase in 0.05 M acetate buffer, pH 5.0 for 20 minutes at RT, and counterstained in Mayer's haematoxylin before mounting for microscopic examination. Histologically normal human skin was used as a positive control, and similarly stained duplicate sections where the primary antibody was omitted and replaced by PBS were used as negative controls.

Immunoreactivity for syndecan-1 was classified by assessing visually the percentage of syndecan-1 positive tumour cells of all tumour cells in the field (I, II, V). First, the percentage of syndecan-1-positive tumour cells of all tumour cells was calculated from 3–5 representative microscope fields per slide (Olympus Optical Company, Tokyo, Japan, ×10 objective, diameter 2.6 mm, area 5.3 mm^2). All selected fields contained at least 100 cancer cells, and necrotic areas and those with marked inflammation were excluded. The immunoreactivity for syndecan-1 was also classified with regard to the intensity of staining (III, V) and cellular localization of staining (V). Staining intensity was classified either as absent (–), weak (+), moderate (++), or strong (+++). Cellular localization of staining was assessed as diffuse cytoplasmic staining, staining of cell-cell contact sites, or both. Assessment of syndecan-1 expression was performed without knowledge of the clinical or survival data.

7.4. Enzyme-linked immunosorbent assay (ELISA)

Peripheral venous blood samples were collected in sterile test tubes, centrifuged at 3,000 G for 10 minutes, and then stored at –20°C (III) or at –70°C (IV,V).

7.4.1. Serum syndecan-1 immunoassay (III–V)

Serum syndecan-1 concentrations were determined using human syndecan-1 ELISA (Diaclone Research, Besancon, France) according to the manufacturer's instructions. The system uses a biotinylated monoclonal B-B4 antibody raised against human syndecan-1. The detection steps include streptavidin-horseradish peroxidase and tetramethylbenzidine as

chromogens. For each analysis 50 µl of serum was used. All analyses and calibrations were carried out in duplicate. The calibrations on each microtitre plate included recombinant human syndecan-1 standards. Optical densities were determined using a microtiter plate reader (Multiscan RC type 351, Labsystems, Helsinki) at 405 nm. The blank was subtracted from the duplicate readings for each standard and sample. The serum concentrations are reported as ng/mL. Syndecan-1 serum levels were determined without knowledge of the survival or other clinical data. No loss of syndecan-1 immunoreactivity was observed when 9 samples were subjected to either 3 or 8 freeze-thaw cycles (P=0.89; Friedman test, III). No significant difference in serum syndecan-1 concentrations was found when cancer patient serum samples stored at –20°C for 4 to 6 years (n=22) and samples stored for 7 to 10 years (n=66) were compared, suggesting that there is no major loss of syndecan-1 immunoreactivity during long-term storage as compared with shorter storage (P=0.18; Mann-Whitney test, data not shown) (III).

7.4.2. Serum bFGF immunoassay (III, V)

Serum bFGF concentrations were determined as serum bFGF immunoreactivity using a quantitative sandwich enzyme immunoassay technique (Quantikine High Sensitivity Human FGF basic Immunoassay; R&D Systems, Minneapolis, MN, USA). The system uses a solid-phase monoclonal and an enzyme-linked polyclonal antibody raised against recombinant human bFGF. For each analysis, 100 µL of serum was used. All analyses and calibrations were performed as duplicate. The calibrations on each microtiter plate were determined using a microtiter plate reader (Multiscan RC type 351; Labsystems, Helsinki) at 490 nm. The blank was subtracted from the duplicate readings for each standard and sample. The concentrations are reported as pg/mL. Serum bFGF levels were determined without any knowledge of the survival or other clinical data. No association was found between serum bFGF concentrations and the duration of storage in serum samples stored at –20°C for 11 to 17 years (Salven et al., 1999).

7.4.3. Serum carcinoembryonic antigen (CEA) immunoassay (III)

CEA was quantitated in serum using an immunofluorometric assay (AutoDELFIA; Wallac, Turku). The detection limit of the assay is 0.2 ng/mL. The upper reference limit is 5 ng/mL.

7.5. Statistical methods

Statistical analyses were performed using a BMDP computer program (BMDP Statistical Software; University of California Press, Los Angeles, CA, USA; I–III) or a StatView 4.02 computer program (Abacus Concepts Inc., Berkeley, CA, USA; IV and V). Cumulative survival was estimated with the product-limit method. The log-rank test was used for comparison of survival between groups. The Brookmeyer-Crowley confidence intervals were computed for the median survival times. Frequency tables were analysed with the X^2 test. Comparison of non-normal distributions was done by computing the Spearman's correlation coefficient, and non-normally distributed parameters between 2 groups were compared with the Mann-Whitney test. The relative importance of different variables on survival was studied using a Cox multivariate proportional hazards model.

8. RESULTS

8.1. Expression of syndecan-1 in carcinomas of the head and neck and the lung (I, II, III, V)

In these studies a total of 363 tumour samples were immunostained using a monoclonal B-B4 anti-syndecan-1 antibody, and 291 of the samples were stained with another antibody, 109–4. A majority (n=354) of the tumours were of the squamous cell type, 6 were adenocarcinomas, and 3 cases were small cell carcinomas (TABLE 1). Most of the tumours showed positive staining for syndecan-1. When the B-B4 antibody was used, from 72% to 94% of the tumours stained positively for syndecan-1, and when the 104–9 antibody was used, 98% (I) and 94% (II) of the samples stained positively. The staining characteristics are shown in (TABLE 3).

The cutoff percentage values in survival analyses varied in each study probably due to the type of antibody used to stain the tissue samples and the variations between tumour types and series. Our policy was to use more reproducible cut-offs such as the median, and the tertiles or quartiles instead of "optimal" percentage values that might have produced smaller P values. When the B-B4 antibody was used, the cutoff percentage values chosen were 50% (median, study I, head and neck carcinomas), 10% (median, study II, lung carcinomas), and 10% (lowest tertile, study V, head and neck carcinomas). In study III on lung carcinomas the syndecan-1 immunoreactivity was also classified with regard to the staining intensity as –, +, ++, or +++. Since antibody 104–9 stained a higher proportion of the samples, the cutoff values chosen were also higher, 80% (lowest quartile, I) and 83% (median, II).

8.1.1. Association between syndecan-1 expression and survival

Strong syndecan-1 expression was associated with favourable overall survival in patients with head and neck cancer (I and V) and among those diagnosed with lung cancer of the squamous cell type (II) (TABLE 3). The 2-year survival rates associated with cancers with a high and a low syndecan-1 expression were 72% vs. 50% (P=0.001), 84% vs. 63% (P=0.026), and 89% vs. 50% (P=0.027) in studies I, II, and V, respectively. When antibody 104–9 was used for detection of syndecan-1, a similar result was found among patients with head and neck carcinoma in study I (66% vs. 47%, P=0.02), but not among patients with lung cancer in study II (P=0.60). When the largest subgroup of patients examined in study I, the laryngeal carcinoma patients, were analysed separately for survival, we found high syndecan-1 expression as detected with the B-B4 antibody to be associated with more favourable prognosis (P=0.04). In study I syndecan-1 expression was an independent

TABLE 3. Syndecan-1 expression in cancer tissue

B-B4 immunostaining	
Squamous cell head and neck cancer	Study I
No. of patients Positive for syndecan-1(N/%) Median value Cutoff value in survival analyses Prognostic factor in univariate analysis[a] Prognostic factor in multivariate analysis[a]	175 165/94% 50% (range, 0–100%) 50% yes: P=0.001 yes: P=0.016
Squamous cell lung cancer	Study II
No. of patients Positive for syndecan-1 (N/%) Median value Cutoff value in survival analyses Prognostic factor in univariate analysis[a] Prognostic factor in multivariate analysis	116 83/72% 10% (range, 0–70%) 10% yes: P=0.026 no
Non-small cell lung cancer	Study III
No. of patients Positive for syndecan-1 (N/%) Scoring scale for expression Prognostic factor in univariate analysis	45 34/76% –/+/++/+++ no
Squamous cell head and neck cancer	Study V
No. of patients Positive staining for syndecan-1 (N/%) Median value Cutoff value in survival analyses Prognostic factor in univariate analysis[a]	27 22/89% 15% (range, 0–95%) 10% (lowest tertile) yes: P=0.027
104–9 immunostaining	
Squamous cell head and neck cancer	Study I
No. of patients Positive for syndecan-1 (N/%) Median value Cutoff value in survival analyses Prognostic factor in univariate analysis[a] Prognostic factor in multivariate analysis[a]	175 172/98% 100% (range, 0–100%) 80% (lowest quartile) yes: P=0.02 yes: P=0.03
Squamous cell lung cancer	Study II
No. of patients Positive for syndecan-1 (N/%) Median value Prognostic factor in univariate analysis	116 109/94% 83% (range, 0–100%) no

[a]High (>cut-off value) syndecan-1 expression associated with favourable overall survival.

prognostic factor in multivariate analysis when antibody B-B4 was used (RR 1.9, 95% CI 1.2–3.1), and also when antibody 104–9 was used (RR 1.7, 95% CI 1.1–2.6).

8.1.2. Correlation of syndecan-1 expression with other clinicopathological variables

In study I head and neck cancer cells were more often positive for syndecan-1 (B-B4) when cancer was well- or moderately differentiated than when it was poorly differentiated ($P<0.0001$). Strong syndecan-1 expression was also associated with a low clinical stage (stage 1–2 vs. 3–4, $P<0.0001$), lack of lymph node metastases (N0 vs. N1-3; $P=0.0006$), and a small primary tumour size (T1-2 vs. 3-4, $P=0.02$). When this series was stained with antibody 104–9, low syndecan-1 expression (≤80%) was strongly associated with a low histological grade ($P<0.0001$) and also with the male gender ($P=0.03$). No other statistically significant associations were found between syndecan-1 expression and clinicopathological factors in this study.

In study II high syndecan-1 expression (>10%, as detected with the B-B4 antibody) was strongly associated with high histological differentiation of lung cancer (Gr. I vs. Gr. II vs. Gr. III, $P=0.001$). A similar result was obtained when staining was done using antibody 104–9 ($P<0.0001$). Patients who were older than the median age (62 years) at the time of the diagnosis of lung cancer had tumours with strong syndecan-1 expression more often than those who were younger ($P=0.016$). No significant association between syndecan-1 expression and the primary lung tumor size, nodal status or stage was found, although smaller tumours tended to be more often syndecan-1 positive in B-B4 staining (T1 vs. T2-4, $P=0.08$). In study III patients who had lung cancer with low syndecan-1 expression (– or +) had lower serum bFGF levels than patients who had lung cancer with a stronger syndecan-1 expression (++ or +++; median, 1.2 pg/mL; vs. median, 3.6 pg/mL, respectively, $P=0.018$). In study V, which comprised a small series of patients who had laryngeal cancer, no significant associations between syndecan-1 expression in tissue samples and the clinicopathological factors examined were found, possibly due to the low statistical power and the type of cancer studied.

8.2. Soluble syndecan-1 (III, IV, V)

The median pretreatment serum syndecan-1 levels in the cancer patients of studies III to V ranged from 41 ng/mL to136 ng/mL. In studies III and IV the lung cancer patients had statistically higher pretreatment serum syndecan-1 levels as compared to the controls who had medium levels of 16 ng/mL (range, from 0 ng/mL to 213 ng/mL) and 17 ng/mL (0–308 ng/mL), respectively ($P<0.0001$, in both cases).

8.2.1. Serum syndecan-1 as a prognostic factor in carcinomas of the head and neck and the lung

In study III that consisted of a series of 184 lung cancer patients, high serum syndecan-1 levels were associated with poor outcome (TABLE 4). When the median serum syndecan-1 ectodomain concentration (41 ng/mL) was taken as the cutoff value, patients with higher than the median serum level had a median survival time of 6 months as compared with 11 months in patients who had serum levels lower than the median ($P=0.003$). When the highest tertile level was used as the cutoff value, the median survival time of the patients with a higher serum level than the cutoff value was only 4 months as compared to 11 months among the rest of the patients ($P=0.0001$). These findings remained similar when the median or upper tertile serum syndecan-1 levels were used as cutoff values among the subsets of patients with NSCLC or SCLC (TABLE 4). In study IV high serum syndecan-1 levels correlated with favourable overall survival when the levels corresponding to the upper tertile (212 ng/mL) or upper quartile (283 ng/mL) were chosen as the cutoff levels (TABLE 4). In study V that comprised 44 patients with squamous cell head and neck cancer, pretreatment serum syndecan-1 level did not correlate significantly with overall survival, but patients whose serum syndecan-1 decreased ≥10% from the pretreatment level had more favourable survival than those whose levels remained stable or increased ($P=0.0069$). In multivariate analyses the pretreatment serum syndecan-1 level turned out to be an independent prognostic factor both in NSCLC (study III, $P= 0.011$, RR 1.8, 95% CI 1.1–3.1) and in SCLC (study IV, $P=0.044$, RR 1.68, 95% CI 1.02–2.77).

TABLE 4. Association of pretreatment serum syndecan-1 level with overall survival in univariate analyses

Lung cancer, local and metastatic (Study III)	**N**	**1-yr survival %**	**5-yr survival %**	**P**
Lung cancer, all				
≤41 ng/mL (median)	94	48	18	
>41 ng/mL	90	33	10	0.003
≤59 ng/mL (upper tertile)	123	49	19	
>59 ng/mL	61	25	7	0.0001
NSCLC				
≤40 ng/mL (median)	69	54	23	
>40 ng/mL	69	36	13	0.0085
≤54 ng/mL (upper tertile)	92	51	22	
>54 ng/mL	46	33	11	0.0038
NSCLC (squamous cell)				
≤40 ng/mL (median)	43	58	23	
>40 ng/mL	39	44	18	0.11
≤54 ng/mL (upper tertile)	55	58	24	
>54 ng/mL	27	37	15	0.029
NSCLC (adenocarcinoma)				
≤38 ng/ml (median)	23	48	22	
>38 ng/mL	21	19	5	0.0067
≤53 ng/mL (upper tertile)	30	43	20	
>53 ng/mL	14	14	0	0.0021
SCLC				
≤44 ng/mL (median)	23	30	4	
>44 ng/mL	23	26	4	0.29
≤84 ng/mL (upper tertile)	31	35	3	
>84 ng/mL	15	13	7	0.041
Small cell lung cancer, mostly metastatic (Study IV)	**N**	**1-yr survival %**	**2-yr survival %**	**P**
SCLC, all				
<136 ng/mL (median)	45	51	20	
≥136 ng/mL	43	44	16	0.44
<212 ng/mL (upper tertile)	59	58	25	
≥212 ng/mL	29	38	3	0.0034
<283 ng/mL (upper quartile)	66	56	22	
≥283 ng/mL	22	36	3	0.0046
Head and neck cancer (Study V)	**N**	**1-yr survival %**	**2-yr survival %**	**P**
<75 ng/mL (median)	22	96	75	
≥75 ng/mL	22	82	73	0.53

8.2.2. Associations between serum syndecan-1 level and clinicopathological factors

Patients with a high Karnofsky's performance status tended to have lower pretreatment serum syndecan-1 values than those with a low performance status (studies III and IV; K≤70 vs. K>70, P=0.0011; K≤70 vs. >70, P=0.021; respectively). In study III high pretreatment serum syndecan-1 level was also associated with a large lung cancer mass (stage IIIB or stage IV disease, P=0.0004). A weak positive association was found between serum syndecan-1 and bFGF levels (P=0.044, Spearman correlation coefficient). No such association was found between the serum syndecan-1 levels and gender, age at diagnosis, histological grade, histological type, or serum CEA levels at the time of lung cancer diagnosis. In study IV patients with high serum syndecan-1 level had frequently higher than normal pretreatment serum lactate dehydrogenase (LDH) levels (P=0.0024). In study V head and neck carcinoma patients with a high pretreatment serum syndecan-1 level tended to have a high pretreatment bFGF serum level (P=0.077; Spearman correlation coefficient).

8.3. Clinical significance of serum bFGF level in carcinomas of the head and neck and the lung (III, V)

The median pretreatment bFGF level in lung cancer patients was 2.2 pg/mL (range, from 0 to 62.6 pg/mL, study III), and there was no significant difference in the serum bFGF levels between patients with NSCLC and those with SCLC (median 2.3 pg/mL; range, from 0 to 62.6 pg/mL vs. median 1.8 pg/mL; range, from 0 to 8.6 pg/mL, respectively; P=0.32). These levels are similar to the levels found in the sera of patients with non-Hodgkin's lymphoma (Salven et al., 2000).

In general, high serum bFGF levels tended to be associated with poor outcome (TABLE 5). When the upper tertile (3.4 pg/mL) was chosen as the cutoff value among lung cancer patients (study III), patients who had levels higher than the cutoff value had worse outcome than those with lower serum bFGF levels (P=0.023). The 1- and 5-year survival rates in these groups were 23% and 7%, and 47% and 14%, respectively. When the associations of serum bFGF with survival were investigated within histological subtypes of NSCLC, high bFGF levels were associated with poor outcome in adenocarcinoma (n=30, median level 4.1 pg/mL used as cutoff value, P=0.0031), but not in squamous cell carcinoma (n=61, P=0.45). No significant association between serum bFGF levels and survival was found among patients with SCLC (n=27, P=0.74). When the serum bFGF level corresponding to the highest tertile (3.4 pg/mL) was used as the cutoff value, patients with a poor performance status and those with NSCLC tended to have high serum levels (P=0.05 and P=0.054, respectively). NSCLC patients with adenocarcinoma had higher levels than those with sq-

uamous cell cancer (P=0.027). Gender, age, stage, serum CEA levels, or the histological grade were not associated with serum bFGF levels. These results need, however, be interpreted with caution due to the relatively small numbers of patients in these analyses.

The median serum bFGF level was 4.6 pg/mL (range, from 0.2 to 63.6 pg/mL) among patients with squamous cell head and neck carcinoma (V). High pretreatment serum bFGF levels were associated with poor overall survival (P=0.043; TABLE 5) and marginally with poor disease-free survival in head and neck cancer (P=0.07). Patients with higher than the median serum bFGF level had an 82% 1-year and a 63% 2-year survival rate as compared to survival rates of 96% and 86%, respectively, among the subset of patients who had a serum bFGF level lower than the median. Pretreatment serum bFGF level did not correlate significantly with any other clinicopathological factor investigated in the series, but the number of patients available for analysis was relatively small (n=44).

TABLE 5. Association of serum pretreatment bFGF level with overall survival in univariate analyses

Lung cancer (Study III)	N	1-yr survival %	5-yr survival %	P
Lung cancer, all				
≤2.2 pg/mL (median)	65	46	14	
>2.2 pg/mL	61	31	10	0.16
≤3.4 pg/mL (upper tertile)	86	47	14	
>3.4 pg/mL	43	23	7	0.023
NSCLC				
≤2.3 pg/mL (median)	51	53	20	
>2.3 pg/mL	48	33	10	0.082
≤4.1 pg/mL (upper tertile)	66	56	21	
>4.1 pg/mL	33	18	3	0.0002
NSCLC, squamous cell carcinoma				
≤2.1 pg/mL (median)	31	52	19	
>2.1 pg/mL	30	43	13	0.43
≤2.8 pg/mL (upper tertile)	41	54	22	
>2.8 pg/mL	20	35	5	0.15
NSCLC, adenocarcinoma				
≤4.1 pg/mL (median)	15	60	20	
>4.1 pg/mL	15	13	0	0.0031
≤5.1 pg/mL (upper tertile)	21	43	14	
>5.1 pg/mL	9	22	0	0.12
SCLC				
≤1.8 pg/mL (median)	14	29	0	
>1.8 pg/mL	13	15	0	0.74
≤2.9 pg/mL (upper tertile)	18	28	0	
>2.9 pg/mL	9	11	0	0.67
Head and neck cancer (Study V)	**N**	**1-yr survival %**	**2-yr survival %**	**P**
SCC of the head and neck				
<4.6 pg/mL (median)	22	96	86	
≥4.6 pg/mL	22	82	63	0.043
<6.3 pg/mL (mean)	31	94	83	
≥6.3 pg/mL	13	77	53	0.034

8.4. Effect of treatment on serum syndecan-1 levels in head and neck carcinoma (V)

Serum syndecan-1 levels decreased after the locoregional treatment of cancer as compared to the pretreatment levels. The median serum level in samples collected 3 months after the treatment was 58 ng/mL as compared to the baseline level of 75 ng/mL (n=41, Spearman rank correlation, $P<0.0001$). Those patients whose serum syndecan-1 level decreased over 10% (n=26) from the pretreatment level had a better overall survival than patients with stable or increasing serum syndecan-1 value (n=16, P=0.0069) with 2-year survival rates of 92% and 51%, respectively. Similarly, patients with decreased (>10%) serum syndecan-1 levels also had higher disease-free survival rates than the rest of the patients (P=0.0091). Although based on a retrospective analysis and a small cohort of patients, these findings suggest that some serum syndecan-1 may originate from the tumour, and that serum syndecan-1 deserves to be further evaluated in monitoring of treatment of head and neck cancer.

A transient rise of the serum syndecan-1 levels was found in the serum samples collected during the second week of the radiotherapy course as compared to the pretreatment levels among patients treated with radiotherapy (n=19, V). Ten of these patients had serum samples taken prior to, during, and after radiotherapy. Among these patients the median pretreatment serum syndecan-1 level was 74 ng/mL, 87 ng/mL during the second week of the radiation therapy course (P=0.0096), and 60 ng/mL 3 months after completion of treatment (P=0.0073 as compared with the baseline). In study V 12 patients relapsed during the follow-up, and a serum sample taken at progression was available from 9 of these patients. The median serum syndecan-1 level was 76 ng/mL (range, 23 to 274 ng/mL) at the time of cancer progression while the median level was 52 ng/mL when measured 3 months after treatment of the primary tumour in these 9 cases (P=0.05), which provides further support to the hypothesis that serum syndecan-1 levels may increase in parallel with head and neck cancer progression.

9. DISCUSSION

9.1. Methodological aspects of syndecan-1 immunoassay and immunohistochemistry

Human syndecan-1 ELISA was used to determine serum syndecan-1 concentrations in studies III to V (Table 5). All analyses and calibration were carried out similarly in each study. However, the median serum syndecan-1 values varied between the studies. In study III that involved 184 lung cancer patients, the median value was 41 ng/mL in the entire series and was similar in the subgroups of NSCLC and SCLC patients (40 ng/mL and 44 ng/mL, respectively). In study IV where the study population consisted only of SCLC patients the median level was higher, 136 ng/mL. The majority of the patients (98%) in study IV had stage III or IV cancer (stage IV corresponds to overtly metastatic disease), while in study III 72% of all the NSCLC patients and 82% of the patients with SCLC had stage III or IV disease. In study V, involving patients with SCC of the head and neck and with relatively a favourable prognosis, the median level was 75 ng/mL. Thus, patients in each study had different histological types of tumour with different sizes, and variable proportions of patients with metastatic disease, which may influence median soluble syndecan-1 values.

Little is known about the influence of long-term storage on serum syndecan-1 values. Sera used in study III had been stored at –20°C, while the sera assessed in studies IV and V had been stored at –70°C. The effect of the storage temperature and duration on syndecan-1 immunoreactivity requires further study. In a series consisting of myeloma patients the reported median value for serum syndecan-1 was 165 ng/mL (Seidel et al., 2000a), which is in the same range as the values measured by us in patients with lung cancer or head and neck cancer. These authors found the median serum syndecan-1 value to be 128 ng/mL, which is higher than the median value of 17 ng/ml and 18 ng/mL found by us in the control groups of studies III and IV. Serum syndecan-1 analyses have thus far been used for a few research purposes only, and little is known how the serum concentrations might vary with respect of age, gender, or in different pathological conditions.

We studied syndecan-1 expression in paraffin-embedded tumour samples by immunohistochemistry using both a mouse monoclonal antibody against human syndecan-1 B-B4 (I–III, V) and a rat monoclonal anti-human syndcan-1 antibody 104–9 (I, II). Although a majority of tumours showed at least some positive staining with either antibody (72% to 94% with B-B4 and 94%–98% with 104–9), detection of syndecan-1 expression was very different with the 2 antibodies. The median percentage values of positive staining ranged from 10% to 50% with B-B4 and from 83% to 100% with 104–9 (Table 3).

9.2. Syndecan-1 expression in tumour samples and its prognostic value

Head and neck carcinoma

SCCs of the head and neck frequently recur at the locoregional sites, and development of second primary tumours is not uncommon (Carter, 1991; Dreyfuss and Clark, 1991). The Tumour-Node-Metastasis (TNM) classification that reflects the tumour volume is probably still the strongest prognostic factor in SCC of the head and neck, while biological markers have had relatively little significance in the management of the disease (Carter, 1991). To this background it is of interest that strong syndecan-1 expression detected by immunohistochemistry has been consistently found to be associated with favourable survival as in a series comprising 29 patients with SCC of the head and neck (Inki et al., 1994c), in another series of 98 patients with SCC of larynx (Pulkkinen et al., 1997) as well as in the present series involving 202 patients with SCC of the head and neck. Syndecan-1 (as detected with B-B4) expression was associated with favourable overall survival in both univariate (I,V) and multivariate (I) survival analyses, and expression was also associated with a small primary tumour size, lack of nodal metastases, and a high histological grade of differentiation (I). Similar results were obtained also with antibody 104-9 (I).

In contrast with the consensus in most other types of human cancer, the value of histological grading as a prognostic factor in SCC of the head and neck is still considered controversial by many. In some studies grading has been found to have prognostic value (e.g. Pera et al., 1986; Wiernik et al., 1991), while others report no such value (e.g. Bundgaard et al., 1992). The present findings were somewhat discordant. In study I the histological grade was a strong prognostic factor in a univariate analysis. No significant association between grading and survival was found in study V, but this series was small in size and consisted mainly of small larynx tumours. Histological grading, though it generally predicts outcome relatively well, is subjective and often difficult to reproduce (Bundgaard et al., 1992). We found syndecan-1 expression to be strongly associated with histological grade (I); well differentiated carcinomas more often showed strong syndecan-1 expression than poorly differentiated ones. Interestingly, in one multivariate analysis syndecan-1 was an independent prognostic factor while histological grade had no independent value (I). This result suggests that syndecan-1 expression may be a stronger prognostic indicator than histological grading, but this needs to be confirmed in future studies.

Lung cancer

Expression of syndecan-1 is often altered in cancer, and it has been suggested that loss of syndecan-1 expression in carcinomas accompanies the malignant phenotype (Inki and Jalkanen, 1996; Sanderson, 2001) although results at variance with this have also been

published (Hirabayashi et al., 1998). As in SCC of the head and neck, histological grading may not be a strong prognostic factor in lung cancer, but several studies show that poor histological differentiation is an adverse prognostic factor in NSCLC (Lipford et al., 1984; Takise et al., 1988; Ichinose et al., 1995). Other commonly accepted adverse prognostic factors in lung cancer include advanced stage and age over 60 at diagnosis, male gender, a poor performance status, and weight loss, but many other factors may also be of importance (Nishio et al., 1996; Pastorino et al., 1997; Adachi et al., 1998; Volm et al., 1998; Tanaka et al., 1999; Junker, 2001; Yuan et al., 2001; Mattern et al., 2002; Jeremic et al., 2003).

Syndecan-1 expression turned out to be a strong prognostic factor in a univariate analysis of the series of 116 patients with SCC of the lung (II). As in head and neck cancer, we found a strong association between syndecan-1 expression and histological grading in lung cancer, whereas no association was found with the clinical stage. In study III we did not detect an association between tissue expression of syndecan-1 and survival. However, we were not able to assess the tissue expression of syndecan-1 in this study in full. The tissue samples available for analysis in this series were often small in size, and were entirely lacking for a large proportion of patients creating the possibility of selection bias. The scale of immunostaining classification used (–/+/++/+++ instead of percentage of positively staining cells) was also crude in this study due to the poorer quality of the tissue starting material available. Since adjuvant therapies are being increasingly used in NSCLC, it is of importance to discover new prognostic factors and predictive factors for chemotherapy and radiotherapy efficacy. In general, the present results suggest that high cell surface syndecan-1 expression is associated with favourable survival in SCC of the lung, and that tissue expression of syndecan deserves further study as a novel prognostic factor in lung cancer.

9.3. Clinical significance of soluble syndecan-1

The function and prognostic significance of serum syndecan-1 in different types of human cancers has not been fully investigated. High levels of shed syndecan-1 have been found to be associated with poor outcome in multiple myeloma (Seidel et al., 2000a; Kyrtsonis et al., 2004), but no other data is available on the role of soluble syndecan-1 as a prognostic factor. After evaluating syndecan-1 expression in head and neck and lung carcinomas by immunohistochemistry we were interested in investigating whether serum syndecan-1 levels would be associated with survival in the same carcinomas.

In our series of lung cancer patients a high pretreatment serum syndecan-1 level was associated with poor overall survival in univariate analyses (III, IV), and had an independent influence on survival in a multivariate analysis in NSCLC (III) and in SCLC (IV). Patients

with a poor performance status (III, IV) and in study III those with a large tumour mass tended to have high syndecan-1 serum levels. Since the performance status tends to decrease in parallel with cancer growth, a poor performance status may also in part reflect the overall tumour volume. In study IV patients with SCLC and a high lactate dehydrogenase level at diagnosis also frequently had a high syndecan-1 serum level. LDH is a strong adverse prognostic factor in SCLC together with the clinical stage and the performance status (Stokkel et al., 1998; Yip and Harper, 2000). In the present series it was not significantly associated with prognosis, suggesting that serum syndecan-1 might be a stronger prognostic factor in SCLC than the lactate dehydrogenase level.

In study V on SCC of the head and neck, pretreatment serum syndecan-1 levels were not associated with overall survival, but the size of the series and the limited number of endpoints prohibits drawing firm conclusions on the prognostic value of pretreatment serum syndecan-1 levels in head and neck carcinoma. However, serum syndecan-1 levels decreased following locoregional treatment of cancer and a decrease in the serum levels following primary cancer treatment turned out to be a prognostic factor; a decrease of 10% or more from the pretreatment level was associated with a favourable outcome. Measurement of syndecan-1 levels might thus be helpful in monitoring for treatment response and selection of patients who might benefit from additional treatments. However, the percent changes in the syndecan-1 serum levels were not in general large, and the factors affecting the normal variation of the syndecan-1 levels remain inadequately studied, suggesting that serum syndecan-1 levels cannot be recommended at present for monitoring of treatment response in the clinical setting.

The origin of shed syndecan-1 is not known. Some of the circulating soluble syndecan-1 might have its origin in the tumour tissue, and our results are in line with this hypothesis. A transient rise in the serum syndecan-1 levels on the second week of radiotherapy is also compatible with this hypothesis, since soluble syndecan-1 might be released from cancer cells undergoing apoptosis as a result from radiation therapy. In a recent study on a series of 50 patients diagnosed with multiple myeloma, a significant decrease in the median serum syndecan-1 levels was found among patients who responded to chemotherapy, whereas no change took place in patients who did not respond to chemotherapy (Janosi et al., 2004). Similarly, Aref et al. (2003) concluded, in a study with 25 newly diagnosed myeloma patients, that soluble syndecan-1 was significantly higher in non-responders to chemotherapy as compared to responders, and a high level of soluble syndecan-1 was a negative prognostic factor. They also found that cellular and soluble syndecan-1 consentrations correlated inversely. In one series of 60 patients with SCC of the oral cavity increased syndecan-1 tissue expression in immunohistochemistry after induction chemotherapy predicted good outcome (Nemeth et al., 2005).

Many soluble putative tumour markers have been evaluated in SCC of the head and neck, but despite some promise most markers neither normalize following treatment nor become elevated at the time of tumour recurrence (Maxim and Veltri, 1986; Mevio et al., 1991; Kimura et al., 2000; Homer et al., 2002; Tartour et al., 2001; Yazici et al., 2001; Shang et al., 2002). An example of such a marker is SCC antigen, the levels of which were associated with disease recurrence in a series of 70 patients with laryngeal cancer (Lachowicz et al., 1999). Another example is cathepsin L, the serum levels of which were evaluated in a series of 42 patients with SCC of the head and neck, and the risk of disease recurrence and death were significantly associated with serum cathepsin L levels (Strojan et al., 2001).

As in SCC of the head and neck, many studies have addressed the clinical value of soluble serum markers in NSCLC and SCLC. Most studies have focused on pretreatment serum levels of the marker (Ardizzoni et al., 2001; Buccheri and Ferrigno, 2002; Laack et al., 2002; Neuner et al., 2002; Pujol et al., 2003; Sasaki et al., 2003), and markers have also been evaluated for monitoring of treatment efficacy. Perhaps the most extensively evaluated serum marker in lung cancer is carcinoembryonic antigen (CEA), which is commonly used to assess treatment response. For example, in one series comprising 297 patients with stage I NSCLC, serum CEA level was found to be a useful predictor of survival, and a persistently high CEA level after treatment was a strong indicator of poor prognosis (Sawabata et al., 2002). In another study on patients with NSCLC survival rate was higher among patients who had elevated serum SCC antigen and who became antigen-negative following resection than among patients who remained antigen-positive (Takeuchi et al., 2003). Studies where serum syndecan-1 is directly compared to CEA and other existing serum markers used to monitor lung cancer treatment are now needed.

9.4. Clinical significance of serum bFGF level

Angiogenesis is essential for tumour growth and dissemination (Folkman, 1990). One of the several growth factors implicated in angiogenesis is bFGF, which is expressed by most tumours (Folkman and Klagsbrun, 1987; Nanus et al., 1993; Ohta et al., 1995; Riedel et al., 2000). Elevated serum bFGF levels have been detected in patients with various types of cancer; in patients with non-Hodgkin's lymphoma, melanoma, lung cancer and myeloma elevated serum bFGF levels correlate with poor prognosis (Graeven et al., 1999; Dietz et al., 2000; Ueno et al., 2001; Ugurel et al., 2001; Brattström et al., 2002; Ruotsalainen et al., 2002; Bentas et al., 2003; Kyrtsonis et al., 2004). In line with most previous studies we found the pretreatment serum bFGF level to be associated with poor outcome in NSCLC (III) and SCC of the head and neck (V).

Pretreatment serum bFGF levels did not significantly correlate with the clinical stage, which is in line with several prior studies (Ueno et al., 2001; Brattström et al., 2002; Homer et al., 2002; Ruotsalainen et al., 2002). However, opposing findings have been reported in renal cell carcinoma, melanoma, and hepatocellular carcinoma, where elevated serum bFGF levels were associated with advanced disease stages and the tumour burden (Dosquet et al., 1997; Poon et al., 2001; Ugurel et al., 2001). In one study bFGF expression as detected by immunohistochemistry, was associated with stage in NSCLC (Takanami et al., 1996).

Pretreatment serum bFGF levels had independent prognostic influence in NSCLC in a multivariate model. In SCC of the head and neck it turned out to be a prognostic factor in univariate analysis. Although we could not perform multivariate analysis in study V due to the limited size of the series, the results suggest that pretreatment serum bFGF level is a prognostic factor both in NSCLC and in SCC of the head and neck.

Serum bFGF levels may decrease after treatment of cancer (George et al., 2002; Ria et al., 2004), supporting the contention that some of the soluble bFGF originates from the tumour tissue. Syndecan-1 functions as a co-receptor of the bFGF receptors, and binds bFGF with less avidity than the light affinity receptors. Presence of increased amounts of soluble syndecan-1 may be associated with an adverse outcome, because elevated levels of soluble syndecan-1 may carry larger amounts of bFGF in the blood circulation and deliver it more effectively to the tumour deposits. The role of syndecan-1 in bFGF signaling is controversial and the literature reports are conflicting. One study identified syndecans to inhibit bFGF signaling, whereas in an other study syndecans were found to promote bFGF signaling (Aviezer et al., 1994; Steinfeld et al., 1996).

10. SUMMARY AND CONCLUSIONS

The main conclusions of the present study are:

1. Decreased expression of syndecan-1 is associated with low histological grade of differentiation and poor outcome in SCCs of the head and neck and in lung cancer. Syndecan-1 is a novel prognostic factor in both cancer types.

2. High serum syndecan-1 levels may be measured in patients with lung cancer or SCC of the head and neck prior to cancer treatment. In general, high serum levels are associated with a poor outcome in lung cancer.

3. Patients with squamous cell head and neck cancer whose serum syndecan-1 levels decrease following cancer treatment may have a more favourable clinical outcome than patients who have stable or increasing serum syndecan-1 levels in longitudinal follow-up.

4. High pretreatment serum bFGF level is associated with poor outcome in patients with NSCLC or SCC of the head and neck.

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