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Insulin-degrading enzyme (IDE) in normal and malignant breast tissues: An immunohistochemical study

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Abbreviations

Ab	antibody
Aß	amyloid beta
AEC	3-Amino-9-Ethylcarbazol
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
APAAP	alkaline phosphatase anti-alkaline phosphatase
ATM	ataxia teleangiectasia mutated
BRCA 1	breast cancer gene 1
BRCA 2	breast cancer gene 2
Caco-2	colon adenocarcinoma cell line
Chicago ab	antibody to full-length IDE
CI	confidence interval
CIPSs	cytosolic insulin-binding proteins
CTCF	CCCTC-binding factor
DNA	desoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERα	estrogen receptor alpha
G_0	gap-phase (cell-cycle)

G_1	gap-phase (cell-cycle)
GH	growth hormone
HER-2	human epidermal growth factor receptor 2
HRT	hormone replacement therapy
IDE	insulin-degrading enzyme
IGF	insulin-like growth factor
IGFBP-3	insulin-like growth factor binding protein 3
IHC	immunohistochemistry
Km	Michaelis-Menten-constant (mol/l)
M16	metalloendopeptidase family 16
maspin	mammary serine protease inhibitor
MCF-7	human breast cancer cell line
n.a.	not available
PAI-1	plasminogen activator inhibitor type 1
PAP	peroxidase-anti-peroxidase method
PDI	peptide disulfide isomerase
PDGF	platelet-derived growth factor
P-gp	p-glycoprotein
Pineda ab	antibody to IDE peptide
PR	progesterone receptor
POX	peroxidase
PTEN	phosphatase and tensin homologue deleted on chromosome ten

RB	retinoblastoma protein
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	room temperature
S-phase	DNA synthesis phase
TBS	tris-buffered saline
TGF-α	transforming growth factor alpha
TNF- α	tumour necrosis factor alpha
tPA	tissue type plasminogen activator
uPA	urokinase type plasminogen activator
vs	versus

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1 Introduction

1.1 Epidemiology of cancer

Cancer is a widespread disease: In 2000 there were an estimated 10 million new cases, 6 million deaths and 22 million persons living with cancer. The most common cancers are, in terms of new cases, lung (1.2 million), breast (1.05 million), colon-rectum (954000), stomach (876000) and liver (564000) (PARKIN, 2004). The annual report to the American nation on the status of cancer demonstrated that the risk of dying from cancer continues to decrease and that the overall cancer incidence rate of both sexes is unchanged (NATIONAL CANCER INSTITUTE, 2005) Cancer death rates from all cancers combined dropped 1,1 percent per year from 1993-2002. The declines in death rates reflect progress in prevention, early detection as well as better treatment. The aetiology of cancer has been studied for a long time. Meanwhile it is well-established that the most important cancer risk factors include specific components of nutrition (e.g. increased consumption of red meat) and smoking as well as other aspects including genetic predisposition (EDWARDS et al, 2005).

1.2 Epidemiology of breast cancer

Among women, breast cancer is the most frequently diagnosed tumour type after non-melanoma skin cancer, and is the second leading cause of cancer death after lung cancer (AMERICAN CANCER SOCIETY, 2005). Every year more than 1000,000 cases are diagnosed world-wide (FERLAY et al, 2000). The annual incidence of breast cancer in Europe is about ~320,000 (31% of all cancers in women) and continues to rise world-wide (BRAY et al, 2002; GOLDHIRSCH et al, 2005). Each year approximately 8-9 % of women contract breast cancer (TUMORZENTRUM MÜNCHEN, 1998). The lifetime risk of any particular woman getting breast cancer is about 1 in 8 although the lifetime risk of dying from breast cancer is much lower at 1 in 28 (THE AMERICAN CANCER SOCIETY, 2005*).* Nevertheless, overall breast cancer mortality has decreased 2.3 % per year in the last 10-15 years mainly because of increased awareness, improvements in cancer screening and novel and more effective therapies (STEWART et al, 2004). Breast surgery is limited by the invasion of operation and the metastasise disease. Chemotherapy and radiotherapy provide treatment modalities not being so invasive but

they are associated with many side effects. However, the mortality for metastatic breast cancer has remained essentially unchanged for the period between 1980 and 2000 (SCHLESINGER-RAAB et al, 2005), even if quite recently some progress could be noted in the treatment of the subgroup of HER-2/new positive breast tumours (HARBECK et al, 2003).

Metastasis is the main cause of death for at least half of breast cancer patients. From node-negative primary breast cancer patients one third of them will have a relapse in a decade because the cancer which had already spread at the moment it was diagnosed remained undetected. Neither the response for breast cancer therapies nor the evolution of the disease can be predicted exactly. Chemotherapy remains an essential first-line treatment component for women who confront a diagnosis of recurrent breast cancer. The application of an adjuvant systemic chemotherapy or antihormones has reduced the number of odds of death by 25% that is an absolute reduction of 4 and 10% in node-negative and node-positive patients, respectively (EARLY BREAST CANCER TRIALISTS´ COLLABORATIVE GROUP, 1992). As many patients with primary breast cancer are over treated, it appears necessary to determine the risk of each patient identifying high risk and low risk patients in order to find an effective treatment (KLIJN et al, 1993). Diminishing the overall tumour burden and thereby controlling disease progression are increasingly important end points with which one can reliably measure the effectiveness of treatment early in the disease. In the last years there is an increasing emphasis on tumour biomarkers that are indicative of or related to cell traits characterising malignancy (DAIDONE, 2004). Tumour-associated biomarkers are useful in cancer diagnostics and screening, determining prognosis, prediction of therapy response and monitoring the course of disease (SCHMITT et al, 2004).

1.3 Risk factors of breast cancer

Breast cancer is a complex disease and a variety of risk factors determine its aetiology and development. Epidemiological studies conducted in different populations distinguish between well-established and probable risk factors for breast cancer. Well-confirmed factors include: age, geographical region (USA and western countries), familial history of breast cancer, genetic factors, cancer in other breast, ionizing

radiation exposure, history of benign breast disease, reproductive events, exogenous hormones, high mammographic breast density, tall stature, lifestyle risk factors (alcohol, diet, obesity and physical activity), high prolactin and insulin-like growth factors (IGF-I) levels. Probable risk factors for breast cancer include high saturated fat and well-done meat intake, polymorphismus in low-penetrance genes and a high socioeconomic status (DUMITRESCU, 2005).

Age, a risk factor for any type of cancer, together with the geographical location (country of origin) is strongly associated with breast cancer risk. The incidence of breast cancer is low before age 25 (less than 10 new cases per 100.000 women) and rises up to 100-fold by age 45 (HULKA, 2001). It can be summarized that cumulative risk of breast cancer increases with age (FEUER, 1993). One possible reason is supposed to be the influence of hormones increasing during the reproductive period. There is a large variation of breast cancer incidence and mortality in different regions of the world depending on environmental and sociocultural factors. In the Far East, Africa and South America breast cancer does not occur as often as in North America and Northern Europe. These facts are linked to genetic differences among populations and/or differences in lifestyle, including diet and environmental exposures. Studies of migrants showed that breast cancer incidence increases in people who move from low-risk areas (i.e. Asian countries) to other regions with higher breast cancer incidence (i.e. US), after only 10 years spent in the adopted country (ZIEGLER et al, 1993). Within one or more generations breast cancer risk of migrants´ descendants is at a similar level as of the native population (MCPHERSON, 2000).

A well-established major risk factor is the family history of breast cancer. This fact is confirmed by studies which estimate the relative risk to get breast cancer with an affected first-degree relative at 2.1% (95% confidence interval (CI) 2.0-2.2). The risk of developing the disease increases with the number of affected relatives and the closeness of their biologic relationship (COLDITZ et al, 1996; PHAROAH et al, 1997).

In addition we also have to take into account the genetic risk for developing breast cancer. As such, specific gene mutations are considered to be another important risk factor for the development of cancer. The majority of breast cancers (approximately

90%) are sporadic cancers which derive from an interaction between low-penetrance cancer susceptibility genes with endogenous and lifestyle risk factors (JOHNSON-THOMPSON, 2000). In contrast there is an increased risk for getting hereditary breast cancer in the case of germ line mutations in high penetrance susceptibility genes like the tumour suppressor genes BRCA1, BRCA2, p53, ATM and PTEN. These mutations make up only 5-10% of breast cancers (EASTON, 1993). Hereditary breast cancers are mainly diagnosed at an earlier age being often multifocal in contrast to sporadic cancers which are bilateral and arise at older age (REBBECK, 1999).

A history of benign breast disease is also known to increase breast cancer risk depending on the stage of benign breast disease. Women with severe atypical hyperplasia have a four to fivefold increased breast cancer risk, with proliferative disease without atypia 1.6-fold to 1.9 fold in comparison with normal breast tissue (MCPHERSON, 2000; LONDON et al, 1992; CARTER et al, 1988). A previous primary breast cancer means a 3-fold to 4-fold increase in risk of a second breast cancer in the contra lateral breast. While the risk of contra lateral breast cancer persists for up to thirty years after the original diagnosis, the median interval between primary breast cancer and contra lateral disease is approximately 4 years.

The number and the timing of different reproductive events in women's life contribute significantly to the risk of breast cancer tumour origin. The hormonal exposure of women to endogenous hormones is influenced by different variables: age at menarche, age at first full-term pregnancy, age of menopause and higher parity. Early menarche (<12 years) implicates such as late menopause that these women are exposed to more regular ovulatory cycles than other women during her lifetime leading to a stronger oestrogen and progesterone exposition. A protective effect has been seen with early first full-term pregnancy and also higher parity (3 or more). In contrast null parity and late age at first full-term pregnancy lead to an increased risk of breast cancer (MCPHERSON, 2000; COLDITZ, 1996).

The intake of hormones as a medication is as important as the influence of endogenous hormones. After menopause hormonal usage is correlated with an increase in breast cancer risk depending on exposure time and whether the oestrogen is used alone or in

combination with progestin (ROSS et al, 2000). Several studies showed that long-term replacement therapy (HRT) may result in the cumulative increase of breast tumours in comparison to those expected in women between age 50 and 70 that never took HRT (COLLABORATIVE GROUP ON HORMONAL FACTORS IN BREAST CANCER, 1997). The use of oral contraceptives leads to a moderate increase of breast cancer risk among long-term users, but 10 or more years after stopping no difference was detectable. Studies demonstrated that the take of combined oral contraceptives results in a higher risk to get breast cancer independent of dose, first dose age and length of application (COLLABORATIVE GROUP ON HORMONAL FACTORS IN BREAST CANCER, 1996).

Lifestyle factors are modifiable risk factors including alcohol consumption, obesity after menopause, diet, weight gain and physical activity. For every 10g-increment (approx. 0,75l-drink) in daily consumption of alcohol the risk of developing breast cancer rises 9% (SMITH-WARNER et al, 1998). Obesity represents a high breast cancer risk factor for postmenopausal women, whereas in premenopausal women it is protective (HUANG et al, 1997). Other factors which are correlated with a rise of breast cancer risk include height and high prolactin levels (HANKINSON et al, 1999; CLEVENGER et al, 2003). Studies demonstrate a relation between high IGF-levels, the anabolic effecter of the growth hormone (GH) and breast cancer in premenopausal women (RENEHAN et al, 2004). In addition, a joint effect of insulin-like growth factors and sex steroids on breast cancer risk in pre- and postmenopausal women was investigated (YU et al, 2003). Recently a relation between high insulin levels and poor outcomes in women with breast cancer has been found (GOODWIN et al, 2002).

1.4 Prognostic factors of breast cancer

Until now all the risk factors with their individual impact on breast cancer have not been able to completely reflect the overall risk for the development of breast cancer. In this case prognostic factors and predictive factors calculating breast cancer development are helpful. The function of prognostic factors is the evaluation of the evolution of the disease. The established prognostic factors include tumour size, axillary lymph nodes status, histological grade and biological tumour features like steroid hormone-receptor status (AGO GRAVENBRUCH, 2002).

The strongest prognostic factor for breast cancer is the number of tumour-infiltrated lymph nodes which correlates directly with the patient's risk of relapse or death. There is also a direct relationship between the size of the primary tumour and the axillary node status (HARBECK et al, 2003). A positive steroid hormone receptor (ERα, PgR) status usually predicts a successful endocrine therapy. But it must be considered that not all these tumours expressing hormone receptors have a clinical relevance. Interestingly, patients with hormone receptor negative tumours reach a complete remission rate of over 30 % after the application of a neoadjuvant cytotoxic therapy and have a shorter survival rate than hormone receptor positive patients who more rarely achieve a complete remission (COLLEONI et al, 2004).

Over the past years several new prognostic factors were detected and evaluated like tumour-associated proteolytic factors (kathepsine and matrix metalloproteinases), cell kinetic factors, and new molecular parameters. Only some have high marker quality providing a much more reliable prognostic quality than other known factors. This is the requisite for being applicable in the routinely diagnosis. These factors have a strong influence on tumour cell growth and aggressiveness (HARBECK et al, 2003). They determine, for example, the ability of tumour cells to spread out of the primary tumour (proteolysis, invasion), to roam in a special direction (adhesion, migration) and to enter healthy tissue over angiogenesis (metastasis). The concentration of these factors in the tumour tissue predicts the probability of relapse of the disease. Two such factors are uPA and PAI-1. They are involved in the (re)modelling of the tumour stroma and the degradation basal membrane and consequently increase tumour cell invasion and metastasis. Both together with the uPA receptor allow the invasion of tumour cells and play an important part in adhesion and migration of the tumour cells (ANDREASEN et al, 1997). High concentrations of uPA and PAI-1 in the primary tumour are accompanied by a higher metastasis risk and a shorter total life span (LOOK et al, 2002). Conversely, nodal negative patients with low levels of uPA and PAI-1 in the primary tumour have a good prognosis and they do not need adjuvant chemotherapy (JÄNICKE et al, 2001). In addition, studies showed that mamma cancer patients with high levels of uPA and PAI-1 show a better response to adjuvant chemotherapy than those with low levels (HARBECK et al, 2002). A simpler detection

of this marker by micro-ELISA or IHC on surgical tumour specimens or using tissue obtained by needle biopsy would be very helpful (GOLDHIRSCH et al, 2005).

Actually the Her-2/neu oncoprotein may be considered to be a new prognostic marker which is overexpressed in 25% of the invasive breast cancers. A Her-2/neu oncoprotein overexpression based on an amplification of the HER-2/neu gene is correlated with a more aggressive evolution of the illness and a downregulation of the hormone receptor status (KONECNY et al, 2003).

Predictive factors e.g. estrogen/progesteron receptor status, menopause status and Her-2/neu-status serve as parameters for providing answers to therapy success or failure**.** Her-2/neu is a receptor being expressed in some breast cancers. A positive Her-2/neu status correlates with the response to a therapy with the humanized antibody Herceptin (Trastuzumab), which is directed against the Her-2/neu-onco-protein. Advanced cancer can also be treated by Herceptin as adjuvant therapy (HARBECK et al, 2003). Studies suggest Her-2/neu over expression correlates with a tamoxifen resistance in contrast to aromatase inhibitors which pretend to be more useful (CARLOMAGNO et al, 1996; ELLIS et al, 2001). For the future a useful method to analyze patients´ tumour tissue would be helpful to select patients which profit from Herceptin therapy.

1.5 Growth factors in breast cancer

Over the past years growth factors and their potential role in cancer risk assessment and prevention have become increasingly important. Growth factors are factors which influence normal cell proliferation and differentiation. Proliferation is defined as the multiplication of the number of cells due to the completion of the cell cycle, whereas growth is the increase in cell mass, although quite often these two terms are also used as synonyms. These so-called growth factors exhibit both classical hormonal characteristics and local tissue influences. In the circulation they occur in large quantities and can be measured. Concentrations of growth factors not only vary across population but also depend on other complex relationships (PARDEE, 1989; AARONSON, 1991). Growth factors make cells in the resting or G_0 phase get into and pass through the cell cycle. Extracellular factors such as growth factors determine whether a quiescent cell will begin to proliferate. They also determine whether a

normal proliferating cell in G_1 will continue to cycle reaching the S-phase or will revert to quiescence. In case there is no growth factor stimulation for a short period of time the cell goes back to the G_0 phase (WESTERMARK and HELDIN, 1985). Cell-cycle events become largely independent of extracellular factors after cells enter into S-phase, which is marked by the beginning of DNA, histone and some enzyme synthesis. In order to transverse the G_1 phase the stimulation by growth factors such as insulin-like growth factor 1 (IGF-1) or insulin has to be maintained for several hours (PARDEE, 1989; AARONSON, 1991).

Cells of most major tissue types are targets for growth factors such as insulin that act through a receptor with intrinsic tyrosine activity. In the G_1 phase oncoproteins and their inhibitors also play an important role. A variety of factors such as cytokines or the tumour necrosis factor (TNF) is able to antagonize growth factors. As a result, cells rapidly die or undergo apoptosis if there is no stimulation by growth factors (WILLIAMS, 1991). The regulation of growth factor levels is considered to be relevant both for normal cell and cancer cell proliferation. Studies comparing cancer cells with normal cells have helped to dissect and identify significant regulatory events. Genetic aberrations of growth factor signalling pathways like in the G1 phase are responsible for the defect switching mechanism between quiescence and proliferation in cancer cells. So cancer cells continue to cycle under conditions that are insufficient for normal cell proliferation. The uncontrolled expression of growth factors contributes to the expansion of malignant cells. Whether breast cancer develops and progresses depends on whether growth-stimulatory or growth-inhibitory stimuli predominate (AARONSON, 1991).

Tumour cell growth is stimulated by a variety of factors. Growth factors, cytokines and hormones interact with membrane receptors initiating a cascade of biochemical signals which results in the activation and repression of various subsets of genes. During the development of breast cancer, estrogens (LAI, 2002) and mitogenic growth factors like the epidermal growth factor family and insulin-like growth factors are significantly involved, whereas inhibitory growth factors like the transforming growth factor-ß family (TGF-ß) prevent the development of breast cancer (GOUSTIN et al, 1986; DICKSON et al, 1991; ROBERTS et al, 1985). Insulin-like growth factor I (IGF-I)

and its main binding protein 3 (IGFBP-3) e.g. are both growth hormone (GH) dependent regulatory peptides that are relevant for tumour cell growth and survival. Studies have demonstrated that higher levels of IGF-I increase the risk of pre-menopausal, but not post-menopausal breast cancer. Higher levels of IGFBP-3 may also predict increased risk of pre-menopausal breast cancer. In addition there is a complex relationship between lifestyle and nutrition factors and circulating levels of IGF-I (RENEHAN et al, 2005).

1.6 Insulin

1.6.1 Structure and function of insulin

Insulin is known as a major hormone in the blood circulation regulating glucose homeostasis and bioavailability as well as protein and lipid turnover. In addition, it also acts as a growth factor and affects nuclear processes by influencing gene expression (HARADA et al, 1993). It is involved in cell proliferation and DNA synthesis of normal and neoplastic cells. Insulin is a molecule with a three-dimensional structure consisting of two chains which are connected by disulfide bonds. The plasma half-life of insulin is very short (4-6 min.) because it has to respond quickly to changes in blood glucose (DUCKWORTH et al, 1998). Intracellular insulin has also been shown to display biological activity (MILLER, 1989). The actions of insulin are classified as short-term (glucose uptake and metabolism), intermediate-term (protein and lipid turnover) and long-term (cell growth and mitogenesis) effects (DUCKWORTH et al, 1998).

1.6.2 Insulin clearance

Insulin is mainly cleared by the liver (SATO, 1991; DUCKWORTH, 1988). About half of portal insulin is extracted during the first-pass transit, but this varies depending on the specific situation. Long-lasting rises in portal insulin levels lead to a decrease in clearance because of receptor down-regulation (BENZI, 1994). In the kidney about 50% of peripheral insulin is removed from the systemic circulation (RABKIN, 1983). Insulin is cleared by two mechanisms: glomerular filtration and proximal tubular reabsorption and degradation (RABKIN, 1984). Kidney cells degrade insulin in the same way the liver does. Even more insulin is cleared by the kidney in the case of insulin-treated patients with diabetes than in normal patients (HYSING, 1989). The

remaining insulin is cleared by other tissues of which muscle plays an important role. For clearing insulin is bound to its receptor, then internalized and finally degraded as in other tissues (DUCKWORTH, 1998).

1.6.3 Cellular insulin uptake

The classic cellular insulin uptake mechanism at physiological insulin concentrations is the receptor-mediated endocytosis. Thereby, insulin interacts with the cell membrane receptor. The receptor ß-subunit is autophosphorylated and therefrom starts a phosphorylation cascade (EXTON, 1991). At high insulin concentrations the fluid-phase endocytosis mediates insulin internalization (HARADA et al, 1992). Insulin uptake and degradation occur in adipocytes, fibroblasts, monocytes, lymphocytes, gastrointestinal cells and many other tissues. Under normal conditions, almost all insulin is degraded intracellularly or at least by membrane processes (Figure1). A recent study suggests that significant amounts of insulin may be cleared and degraded extracellularly in wounds (SHEARER et al, 1997).

1.6.4 The role of insulin in the nucleus

The internalization of insulin has been demonstrated in several studies. It is meanwhile well-established that insulin accumulates in the cell nucleus (GOLDFINE, 1976; HEYNER et al, 1989; SOLER and THOMPSON, 1989). Moreover, insulin binds to the nuclear matrix (THOMPSON et al, 1989), an active site of gene transcription (BEREZNEY et al, 1991). Nuclear accumulation of insulin is energy-independent but depends on time and temperature (SMITH et al, 1990). Furthermore, a study has shown that the internalization of insulin and its interaction with the cell nucleus leads to increased RNA and protein synthesis (MILLER et al, 1988). OSBORNE and BOLAN demonstrated in 1976 that DNA, RNA and protein synthesis in human breast cancer maintained in long-term tissue culture can be stimulated by physiological insulin concentrations. They suggested that the MCF-7 cell line provides a model for the study of insulin action and mechanism of growth regulation in breast cancer.

The analysis of insulin´s amino acid sequence (in the B-chain) led to the prediction that insulin may bind to the nuclear tumour suppressor retinoblastoma protein (RB) (RADULESCU and WENDTNER, 1992). The first experimental support for this prediction

was provided three years later by showing that the putative RB binding site synthesized as a peptide recognizes full-length insulin in vitro (RADULESCU, 1995a). Consistent with the nucleocrine hypothesis (RADULESCU, 1995b), it was then shown that insulin colocalizes with RB in the cell nuclei of HepG2 human hepatoma cells and that it contacts, as predicted, the B-region of the RB pocket (RADULESCU et al, 2000). This interaction between a growth factor and an anti-oncoprotein in living cells was found to correlate with an increase in cell proliferation (RADULESCU et al, 2000). RB is a pivotal anti-oncoprotein which controls cell cycle progression and serves different proteins with growth regulatory activity as a target (SHERR, 1996).

1.6.5 Cellular insulin degradation

In addition to its interactions with the cell membrane-associated insulin receptor and with RB in the nucleus (cf. above), insulin interacts with cytosolic insulin-binding proteins (CIPSs) like protein disulfide isomerase and the insulin-degrading enzyme (IDE) (PROZOROVSKII et al, 2003). Cellular insulin degradation is due to a sequential process with an initial reductive cleavage of the molecule and subsequent proteolysis of the separate chains. Intracellular degradation of insulin is initiated in endosomes. IDE recognizes the three-dimensional configuration of the substrate and produces specific cleavage sites in the B-chain of the insulin molecule.

Figure 1 shows the major and minor cleavage sites in insulin produced by this enzyme.

Figure 1: Cleavage sites of insulin

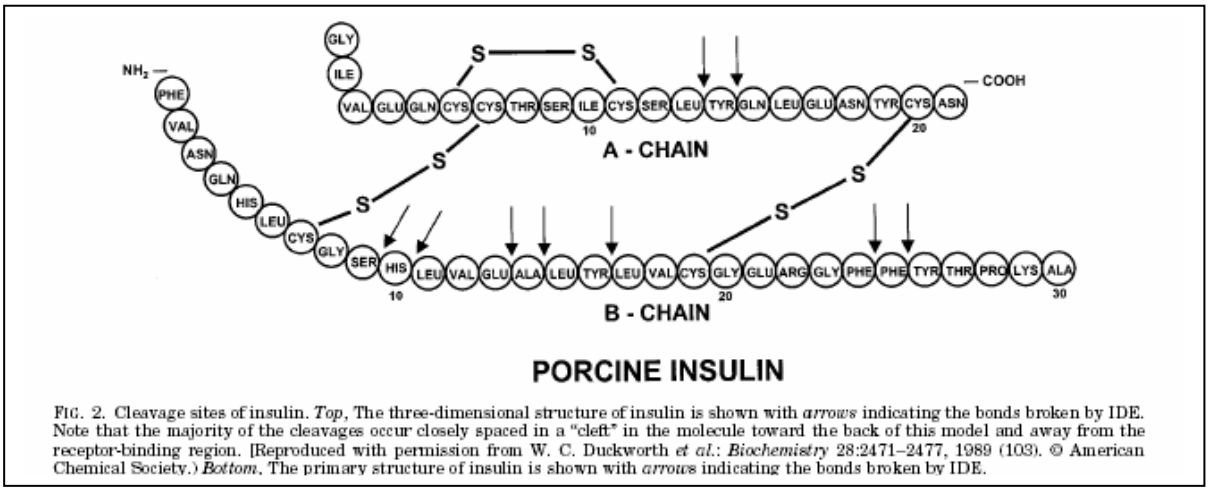


FIG. 2. Cleavage sites of insulin. *Top*, The three-dimensional structure of insulin is shown with *arrows* indicating the bonds broken by IDE. Note that the majority of the cleavages occur closely spaced in a "cleft" in the molecule toward the back of this model and away from the receptor-binding region. [Reproduced with permission from W. C. Duckworth *et al.*: *Biochemistry* 28:2471–2477, 1989 (103). © American Chemical Society.) *Bottom*, The primary structure of insulin is shown with *arrows* indicating the bonds broken by IDE.

Duckworth et al, 1998

Insulin's disulfide bonds are further reduced by the disulfide isomerase (PDI), resulting in a a-chain and several b-chain fragments (SEABRIGHT and SMITH, 1996). Then the insulin fragments are cleaved by different proteolytic systems, including lysosomes. The initial degradation products are very similar from cell type to cell type. By removing insulin it is possible to reduce the reaction of the cell to the hormone but the degradation may also influence insulin action (DUCKWORTH et al, 1998).

Insulin degradation is mainly performed by the cytosolic insulin-degrading enzyme (IDE). It has been demonstrated that inhibiton of cytosolic IDE activity by 1.10 phenanthroline, a zinc chelator, increases nuclear accumulation of insulin (HARADA et al, 1993). By controlling the translocation of insulin to the cell nucleus, IDE is able to modify insulin regulated nuclear events like cell growth and gene trancription. Recently there have been suggestions that insulin interaction with the nucleus and degradation by IDE may play a more direct role in generating insulin effects. IDE activates proteasome and steroid receptors by increasing proteasome proteolytic activity and increasing steroid receptor binding to DNA. Since inhibition of protein degradation, fatty acid oxidation and steroid action are biological effects of insulin these findings raise the possibility that a direct cytosolic interaction of internalized insulin with IDE could be involved in these cellular effects (DUCKWORTH et al, 1998).

1.6.6 Insulin and breast cancer

Several studies have investigated the insulin dependency of breast carcinomas. Specifically, the presence of insulin in breast cancer tissue was shown by immunohistochemistry and RIA (radioimmunoassay). Based on the proven role of insulin as a breast mitogen involved in the initiation and the promotion of tumour growth, it was suggested that in the future the immunohistochemical localization of specific hormone markers may be useful for breast cancer staging, prognosis, detecting spread and distribution of metastatic cells and response to therapeutic regimens (SPRING-MILLS et al, 1984; CASTRO et al, 1980). Furthermore, there is evidence for an overexpression of the insulin receptor in breast cancer tissue (PEZZINO et al, 1996; PAPA et al, 1990). The immunohistochemical detection and the quantification of insulin by RIA in breast cancer tissue initially reported by Castro et al. could be

reproduced by RADULESCU et al. by means of a non-radioactive immunoassay (RADULESCU et al, 2007).

1.7 Insulin-degrading enzyme (IDE)

1.7.1 Structure and function of the insulin-degrading enzyme

Most studies have identified IDE as the main enzyme to process and degrade insulin in cell homogenates. IDE (insulin-degrading enzyme) is a ~110-kd thiol zinc-sensitive metalloendopeptidase belonging to the M16 family because of its characteristical catalytic centre His-Xaa-Xaa-Glu-His (DUCKWORTH et al, 1998). IDE´s main functions are peptide hydrolysis and zinc binding. Although insulin has the highest affinity (based on apparent Km) a number of other peptides can be degraded by IDE (Table 1 DUCKWORTH et al, 1998). These include e.g. glucagon, IGF-II, atrial natriuretic peptide (ANP), transforming growth factor-a (TGF-alpha) and β-Amyloid peptide. IDE cleaves small proteins such as insulin, Aß, Amylin, atrial natriuretic factor and calcitonin which sharing the propensity to form ß-pleated sheets.

IDE is not a general peptidase since a wide variety of other peptides and proteins are not affected by the enzyme. The selectivity of the enzyme has led to the conclusion that substrate recognition depends on the three-dimensional features of the peptide rather than on the proteolysis of specific peptide bonds. Because of the multiple forms and other factors such as the formation of complexes the general characteristics of IDE have been the subject of controversy with different laboratories reporting different apparent molecular weights, Km values, PH optima, inhibitor susceptibility and other properties. Before its degradative function was appreciated IDE was considered to be an intracellular receptor for insulin and other growth factors. IDE was isolated as an intracellular receptor for insulin and related growth factors in Drosophila (GARCIA, 1989). Cellular and endosomal degradation of IDE reacts to modifiers of IDE activity. The injection of monoclonal antibodies to IDE inhibits the intracellular degradation of insulin (SHII and ROTH, 1986). The degradation of insulin by IDE depends on the amount of IDE. These facts suggest that IDE plays the most decisive role for insulin degradation (DUCKWORTH et al, 1998).

Table 1: Reported degradation functions of IDE

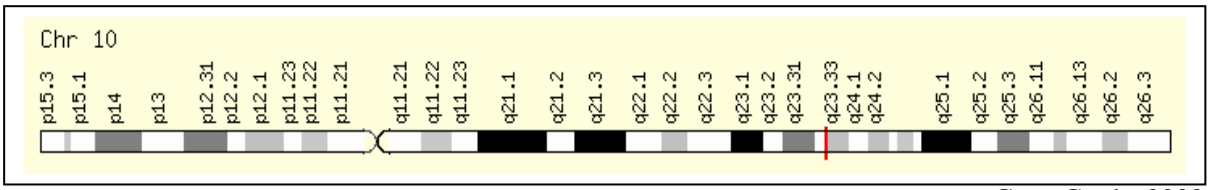
Function	References
Binding and complete degradation	
Aß (ß-Amyloid peptide)	KUROCHKIN AND GOTO, 1994; MCDERMOTT AND GIBSON, 1997; QIU ET AL, 1997; KUROCHKIN ET AL, 2001
Amylin	BENNETT, DUCKWORTH AND HAMEL, 2000 KUROCHKIN ET AL, 2001
ANP (atrial natriuretic peptide)	MULLER ET AL, 1991
ANF (atrial natriuretic factor)	BENNETT, DUCKWORTH AND HAMEL, 2000 KUROCHKIN ET AL, 2001
Glucagon	DUCKWORTH AND KITABCHI, 1974
IGF-II (insulin-like growth factor)	MISBIN ET AL, 1983; ROTH ET AL, 1984; MISBIN AND ALMIRA, 1989
Insulin	DUCKWORTH, 1974; DUCKWORTH, 1988
TGF-α (transforming growth factor)	GEHM AND ROSNER, 1991; HAMEL ET AL, 1997
Oxidatively damaged haemoglobin	BECKER AND ROTH, 1995
Calcitonin	BENNETT, DUCKWORTH AND HAMEL, 2000 KUROCHKIN ET AL, 2001
Thiolase-cleaved leader peptide	AUTHIER ET AL, 1995
Binding and limited degradation	
Proinsulin	KITABCHI ET AL, 1971
IGF-I (insulin-like growth factor)	MISBIN ET AL, 1983; ROTH ET AL, 1984; MISBIN AND ALMIRA, 1989
EGF (epidermal growth factor)	GARCIA ET AL, 1989
Binding	
TGF-ß,	GARCIA ET AL, 1987
nerve growth factor,	GARCIA ET AL, 1987
PGF (platelet-derived growth factor)	GARCIA ET AL, 1987

Duckworth et al, 1998

1.7.2 Genetic background of the insulin-degrading enzyme

The human and mouse IDE genes have been mapped to chromosome 10 and chromosome 19, respectively. The IDE gene appears to be a single, complex gene which may be differentially expressed and developmentally regulated (Affholter et al, 1990).

Figure 2: Gene Card for gene IDE



Gene Cards, 2003

Several different transcripts of the IDE gene have been found in different organisms and different tissues, and it is probable that this gene produces various proteins (KUO et al, 1993; KUO et al, 1994).

1.7.3 Distribution of the insulin-degrading enzyme

IDE is located in both insulin-sensitive and insensitive tissues. It can be detected in several subcellular compartements like endosomes, mitochondria and peroxisomes (DUCKWORTH et al, 1998). The expression of IDE in peroxisomes is explained by its peroxisomal-targeting sequence (Ala-Lys-Leu) at the C-terminus (CHESNEAU et al, 1997) while mitochondria contain a novel isoform of IDE because of its special N-terminal mitochondria targeting sequence. The multiple functional sites where IDE is found support a multifunctional role for this enzyme. In the cell we find IDE principally in cytoplasm, but in different cell types it can be secreted into the extracellular space or correlated with the cell-surface (LEISSRING et al, 2004). The role of IDE in tumour cells still remains unknown and has been poorly investigated. IDE was located by immunohistochemistry in a human colon adenocarcinoma cell line (CHANG et al, 1996).

1.7.4 Recent findings

Interestingly, IDE is also able to degrade Aß (amyloid ß-protein), which accumulates in the brain of Alzheimer patients (MCDERMOTT and GIBSON, 1997) ß-Amyloid shares a common structural property with other IDE substrates like insulin, glucagons, amylin (BENNETT, 2000) and ANF (atrial natriuretic factor) in that all of these peptides can form ß-pleated sheet-rich amyloid fibrils. A genetic linkage and allelic association between the IDE gene locus and late-onset Alzheimer´s disease was reported (EDLAND et al, 2003; PRINCE et al, 2003). These facts underline the importance of IDE function and suggest a therapeutic role in the treatment of Alzheimer disease.

Moreover, a link was also found between type II diabetes mellitus and IDE. There has been reported a genetic association between the IDE gene and type II diabetes mellitus (KARAMOHAMED et al, 2003). Two missense mutations in IDE decrease the ability of IDE to degrade insulin and lead to glucose intolerance (FAHRAI-RAD et al, 2000). These facts underline that there is a significant connection between IDE and type II diabetes mellitus or between IDE and hyperinsulinaemia (RAZAY and WILCOCK, 1994).

Patients with diabetes have a great probability of developing ulcers. Recent advances in wound treatment include topical growth factor therapy. Growth factors like insulin are present in low concentrations in wound fluid which is partially attributed to degradation by IDE and other proteases (DUCKWORTH et al, 2004). Recently it was found that the growth factor insulin which has a long tradition of use in the topical treatment of wounds is degraded extracellularly in wounds by IDE. The regulation of the glucose levels as well as the decrease of the degradation of IDE by insulin in the wound fluid should therefore play an important role when choosing an effective therapy (SHEARER, 1997).

2 Aim of the study

Insulin degradation is mainly performed by cytosolic IDE (HARADA et al, 1993). IDE activates proteasome and steroid receptors by increasing proteasome proteolytic activity and increasing steroid receptor binding to DNA (KUPFER et al, 1993; KUPFER et al, 1994). Since inhibition of protein degradation, fatty acid oxidation, and steroid action are biological effects of insulin these findings raise the possibility that a direct cytosolic interaction of internalized insulin with IDE could be involved in these cellular effects. It has been further demonstrated that inhibition of cytosolic IDE activity by 1,10 phenanthroline, a zinc chelator, increases nuclear accumulation of insulin. By controlling the translocation of insulin to the cell nucleus, IDE is able to modify insulin regulated nuclear events like cell growth and gene transcription. Recently there have been suggestions that insulin's action with the nucleus and degradation by IDE may play a more direct role in generating insulin effects (DUCKWORTH et al, 1998). In this context, the insulin-RB interaction (RADULESCU et al, 2000) has provided a first specific role for insulin in the nucleus.

Based on these findings, the working hypothesis for the present study has now been that IDE acts as a tumour suppressor by inhibiting the translocation of insulin from the cytosol to the nucleus and thereby blocking insulin's binding and inactivation of the nuclear tumour suppressor RB (RADULESCU, unpublished observation, 1994). Therefore, we examined by immunohistochemistry whether there is a difference in the expression of IDE in normal breast tissues vs. (non-metastatic) breast cancer specimens. The expectation has been that, similar to established tumour suppressors, there is either a complete loss of the expression of IDE (at the gene and/or protein level) or an up-regulation of the IDE protein (responding to an increase in the intracellular level of the growth factor insulin in the neoplastic breast tissues) depending on the stage of the tumor disease.

Specifically, in this study we have aimed to establish a first immunohistochemical procedure for the detection of IDE in normal and malignant tissues. For IDE localization antibodies of IDE were produced by immunizing rabbits and chicken with either recombinant or synthetic sequences of IDE and isolating antibodies of their sera.

The objective of the present study was to define the localization of IDE, including the staining intensity and distribution in normal breast tissues vs. breast cancer specimens. IDE expression in breast tumour tissue has been was also correlated to the TNM status and the presence or absence of steroid hormone receptors.

3 Materials and methods for Immunohistochemistry

3.1 Materials

3.1.1 Patient collective

For this study paraffin blocks of formalin-fixed non-pathologic liver and kidney specimens as well as of 24 normal mammary glands and 23 breast carcinomas were provided from the files of the Department of Pathology of the rechts der Isar Hospital of the Technical University Munich (Munich, Germany). Samples of breast tissue were obtained at mastectomy from: 24 women with non pathological breast tissue and 23 women with breast cancer classified according to their grading: fourteen grade 3 (G3), eight grade 2 (G2) and one grade 1 tumor (G1)

These tissue samples were classified according to clinical (history), histomorphological (primary tumour size, nodal status, metastasis, grading) histological grading and tumour biological criteria (steroid hormone receptor status) as well as to the evolution of the illness (prognosis) and therapy response (prediction).

The exact method for IDE immunohistochemistry was established in samples of kidney, liver, placenta, muscle, cutis and brain before applying the method on breast tissue samples.

3.1.2 Tissue processing and fixation

For fixation breast tissues obtained from surgery are placed for fixation in a buffered formalin solution (4% paraformaldehyde in 0.1M phosphate buffer) as routine fixative for immunohistochemistry. To ensure the preservation of tissue architecture and cell morphology, prompt and adequate fixation is essential. However, inappropriate or prolonged fixation may significantly diminish the antibody capability. In these cases a pre-treatment with proteolytic enzymes which degrades the exceeding aldehydes and liberates antigens is recommended. After 6-12 hours breast tissues are subsequently placed in graded alcohol and xylol for dehydration, followed by a paraffin bath. Finally tissues are embedded in a fluid paraffin-plastic cassette, the paraffin temperature not being allowed to exceed 60°C.

3.1.3 Preparation of paraffin sections

The paraffin-block is cut by means of a microtome into 2µm thin layers. After placing the 2µm sections in a water bath they are mounted on a glass slide pre-treated with aminosilan and dried at 37°C over night. Before they can be used for immunohistochemistry they must be placed in a incubator for two hours.

3.1.4 Antibodies

For immunohistochemical determination of IDE in tissue samples the following antibodies were applied:

- polyclonal recombinant anti-IDE-antibody from rabbit (ROSNER, Chicago, USA) directed against full-length IDE (produced according the method of CHESNEAU and ROSNER) (see table 1)
- polyclonal anti-IDE-antibodies from rabbit against synthesized IDE-peptide p15 (YKEMKAVDAPRRHK) (PROF. M. SCHMITT, Munich, Germany) (see table 2)
- polyclonal anti-IDE-antibodies from chicken against synthesized IDE-peptide p15 (YKEMKAVDAPRRHK) (PROF. M. SCHMITT, Munich, Germany) (see table 2)

They were produced as follows:

An antigen is gained from serum, urine or tissue and cleaned using various different procedures. Then the antigen is injected to an animal. This animal must be from a different species than the one from which the antigen was obtained. This animal recognizes the extraneous antigen and produces antibodies which are specifically directed against this antigen. The production of antibodies starts within 20 minutes after the injection although detectable amounts of antibodies can only be determined 5 or 10 days later. In intervals of 15 days small samples of blood are extracted and pooled. Booster injections (i.e. stimulating subsequent injections) of the antigen are required once a month to stimulate a steady production of antibodies. So-called B-lymphocytes produce antibodies directed against this antigen. B-cells can only produce antibodies against an antigen epitope. As many B-cells produce antibodies against each epitope, a polyclonal antibody (i.e. formed by many cells) is formed. The anti-

IDE-peptide antibodies are different in their grade of purification and the time elapsed since the immunisation.

Table 2: Antibodies against full-length IDE

1st Antibody	Dilution	2nd Antibody	Pre-treatment	Method
rabbit polyclonal (Prof. Rosner Chicago)	1/50,1/100, 1/200, 1/250, 1/300, 1/400	mouse anti rabbit	Pressure cooking in citrate buffer (pH 6.0)	POX
mouse monoclonal (Covance, Inc.)	1/50,1/100, 1/200, 1/250, 1/300, 1/400	Dako Kit (K5000) (Bottle A)	Pressure cooking in citrate buffer (pH 6.0)	APAAP

Hufnagel, 2005

From rabbit we got polyclonal not-purified IDE- peptide antibodies of the following days elapsed since immunisation: 61, 90, 120 and 160. From chicken we produced polyclonal not-purified IDE- peptide antibodies of following days: 61, 90, 120 and 185. To avoid unspecific reactions they were purified (see table 2).

Table 3: Antibodies to IDE peptide (produced for Prof. M. Schmitt, Munich by Pineda GmbH, Berlin)

Animal	Day	Purification	Dilution	Pre-treatment	Method
Rabbit 1	61	not-purified		-	POX/AEC
	90	not-purified	1/100,1/500	-	POX/AEC
	120	not-purified	1/100,1/500	-	POX/AEC
	160	not-purified	1/650,1/800,1/1000	-	POX/AEC
	160	Purified	1/50,1/100	-	POX/AEC
Rabbit 2	61	not-purified		-	POX/AEC
	90	not-purified	1/100,1/200,1/300, 1/500	-	POX/AEC
	120	not-purified	1/100,1/200,1/300,1/ 400,1/500	-	POX/AEC
	160	not-purified	1/650,1/800,1/1000	-	POX/AEC
	160	Purified	1/50,1/100	-	POX/AEC
Chicken	61			pressure cooking	POX/AEC
	90	not-purified	1/100,1/500	pressure cooking	POX/AEC
	185	not-purified	1/100,1/500	pressure cooking	POX/AEC
	185	Purified	1/50,1/100,1/200, 1/1000	pressure cooking	POX/AEC
	120	not-purified	1/50,1/100,1/200, 1/500	pressure cooking	POX/AEC

Hufnagel, 2005

For determining the specificity of immunohistochemical staining recombinant IDE and synthesized IDE were provided. Antibodies were incubated with recombinant IDE and synthesized IDE before covering slides with them. An excess of these reagents should reduce the binding of the antibodies to intracellular IDE.

3.2 Methods

Immunohistochemistry was done in the Clinical Research Unit of the department of obstetrics and gynaecology of the Technical University of Munich. The immunohistochemical staining was evaluated by Dr. WEIRICH of the Institute of Pathology of the Technical University of Munich.

There are four methods of immune peroxidase staining for the localization of cellular antigens. The direct, indirect, PAP- and Avidin-Biotin- method have advantages and disadvantages which must be considered. Immune peroxidase procedures offer the possibility to visualize cell components in different tissue sections like paraffin slides. According to the specific antigen it is possible to determine the type of cell producing the substance in normal and neoplastic tissue and the amount of the substance. In addition cells can be identified and differentiated. Antibodies as well as the enzyme peroxidase are used for immune peroxidase method.

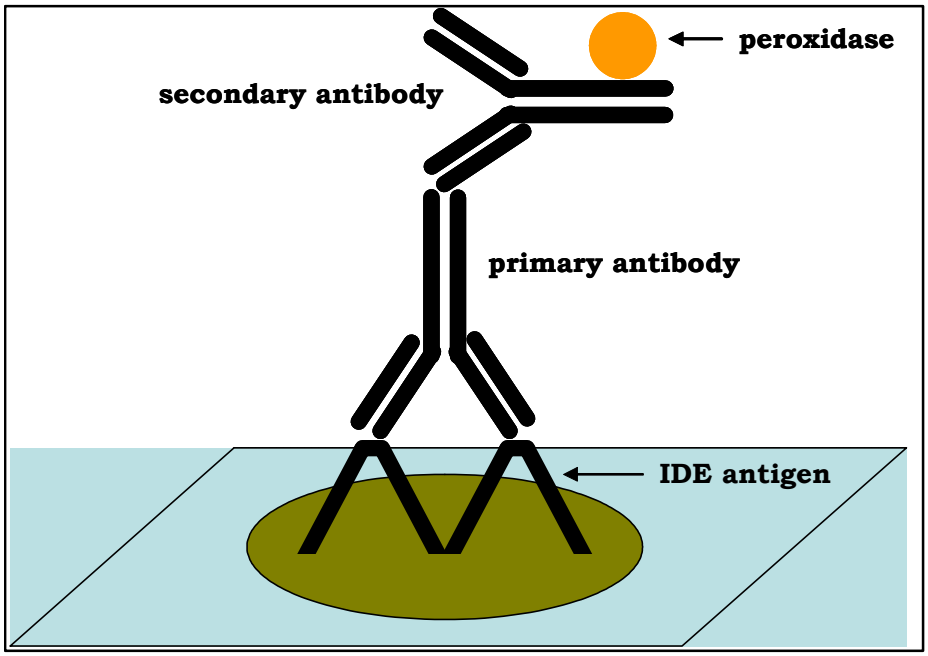
The advantages of the enzyme peroxidase are:

- it is small and does not impede the binding of antibodies to the adjoining points
- it is easy to obtain in a highly purified form, contributing to diminish the risk of contamination.
- it is very stable and remains unaltered during its production, storing and application
- only small amounts of peroxidase are in the tissue itself and this endogenous peroxidase activity can be easily suppressed by immersing paraffin slides into a H2O2 bath
- there are a lot of chromo genes which form a colour product with peroxidase which precipitates at the location of the antigen.

3.2.1 Indirect immunoenzyme method

For immunohistochemistry we applied the indirect method. This method involves an unlabeled primary antibody (first layer) which binds to the tissue antigen. For the localization of the binding an enzyme-labelled secondary antibody (second layer) which binds to the primary antibody (now the antigen) is then applied. The second layer antibody may be labelled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called indirect immunoenzyme method. In the case that the primary antibody is made in rabbit or mouse, the secondary antibody must be directed against rabbit or mouse immunoglobulins, respectively. A substrate-chromogen solution is added to localize the reaction.

Figure 3: Indirect immunoenzyme method



Hufnagel, 2006

The advantage of this method in comparison to the direct method is that a lot of primary antibodies from the same species can be used together with the same labelled secondary antibody. If there is a peroxidase labelled secondary antibody this method can be used with each primary antibody.

The disadvantage of this method is that it takes a lot of more time than the direct method and unspecific reactions are much more probable. The procedure is also more sensitive than the direct method as several secondary antibodies are likely to react with a number of different antigenic sites on the primary antibody thus amplifying the signal as more enzyme molecules are attached per each target site.

3.2.2 Immunohistochemical protocols

Immunohistochemical protocol for the anti-full-length-IDE antibody

Sections from normal and tumour breast tissues are mounted on slides for immunohistochemical analysis as described above.

Deparaffinization and rehydration

The formalin-fixed and paraffin-embedded sections are deparaffinized and rehydrated in a descending grade alcohol series (two times in a xylol bath for 10 min, two times in

a 100% alcohol bath for 5min., once in a 96% alcohol bath for 5 min and once in a 70% alcohol bath for 5min.).

Washing

The previous procedure is followed by the washing of the sections with a Tris-buffered saline solution (TBS 0.005M Tris, pH 7.6 [SIGMA, Munich, Germany]) for 5 min renewing the buffer after half of the time.

Antigen Retrieval

To allow antigen retrieval we tried out 3 different pre-treatment methods like pressure-cooking in citrate buffer (sodium citrate buffer, pH 6.0 [SIGMA C-1909, MW 210.1, Munich, Germany]), vapour-cooking in buffer (ph 6.0) and protease. The best staining results were obtained with pressure-cooking in citrate buffer (sodium citrate buffer, pH 6.0[SIGMA, Munich, Germany]) for 4 min.

Blocking

Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide (45 ml aqua dist., 5 ml H_2O_2 30% [MERCK, Darmstadt, Germany]) for 20 min at room temperature.

Washing

The sections were washed with water, then placed in a Tris-buffered saline solution (TBS: 0.005M Tris, pH 7.6[SIGMA, Munich, Germany]) for 5 min renewing the buffer after half of the time.

Primary antibody

The sections were covered with a rabbit polyclonal antibody that has been raised against recombinant human IDE (CHESNEAU & ROSNER 2000) whereby this antibody was employed at a 1:200 dilution in antibody diluent (DAKO, Hamburg, S2022) and incubated overnight at 4°C. The positive control liver or kidney section was also treated with the primary antibody whereas the negative control kidney or liver section is not.

Washing

Sections were washed with a Tris-buffered saline solution (TBS: 0.005M Tris, pH 7.6[SIGMA, Munich, Germany]) for 5 min renewing the buffer after half of the time.

Secondary antibody

The tissue sections were then incubated with the peroxidase (POX)-labeled mouse anti-rabbit antibody (DIANOVA, Hamburg, Germany cat. # 211-035-109) - diluted (1:50) in antibody diluent (DAKO, Hamburg, S2022) - for 30 min.

Washing

Sections were washed with a Tris-buffered saline solution (TBS: 0.005M Tris, pH 7.6[SIGMA, Munich, Germany]) for 5 min renewing the buffer after half of the time.

AEC substrate

Peroxidase activity was revealed with the chromogen AEC (DAKO, Hamburg, Germany, K3464) for 10 min at room temperature. Nuclei of cells were subsequently counterstained in Mayer´s acid hematoxylin for 90 sec, rinsed under running tap water for 5 min, transferred to distilled water and finally mounted in Kaiser´s glycerol gelatin (MERCK, Darmstadt, Germany, cat. # 1.09242).

Immunohistochemical protocol for the IDE (peptide)-Antibodies

Deparaffinization and Rehydration

The formalin-fixed and paraffin-embedded sections are deparaffinized and rehydrated in graded alcohols (two times in a xylol bath for 10min., two times in a 100% alcohol bath for 5min., once in a 96% alcohol bath for 5min. and once in a 70% alcohol bath for 5min.).

Washing

The previous procedure is followed by the washing of the sections with a Tris-buffered saline solution (TBS 0.005M Tris, pH 7.6 [SIGMA, Munich, Germany]) for 5 min. renewing the buffer after half of the time.

Antigen retrieval

To allow antigen retrieval sections which were incubated with the primary IDE (peptide)-antibodies from chicken were pressure-cooked in citrate buffer (sodium citrate buffer, pH 6.0 [SIGMA, Munich, Germany]) for 4 min. Sections which were incubated with the primary IDE (peptide)-antibodies from rabbit did not need a pre-treatment to allow antigen retrieval.

Blocking

Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide (45 ml aqua dist., 5 ml H2O2 30% [MERCK, Darmstadt, Germany]) for 20 min. at room temperature.

Washing

The sections are washed with water, then placed in a Tris-buffered saline solution (TBS: 0.005M Tris, pH 7.6 [Sigma, Munich, Germany]) for 5 min. renewing the buffer after half of the time.

Primary antibody

The sections are covered with the polyclonal IDE (peptide)-antibodies from rabbit and from chicken (SCHMITT, Munich) respectively diluted with antibody diluent (SIGMA, Munich, Germany) and incubated over for 1 hour at RT. The positive control liver or kidney section is also treated with the primary antibody whereas the negative control kidney or liver section is not.

Washing

Sections are washed with a Tris-buffered saline solution (TBS: 0.005M Tris, pH 7.6[SIGMA, Munich, Germany]) for 5 min. renewing the buffer after half of the time.

Secondary antibody

This is followed by incubation of the sections with the peroxidase labelled mouse anti rabbit (POX) antibody (DAKO, Hamburg) and with the peroxidase labelled mouse anti

chicken IGG antibody (DAKO, Hamburg) respectively diluted (1:50) and (1:500) in antibody diluent (SIGMA, Munich, Germany) respectively for 30 min.

Washing

Sections are washed with Tris-buffered saline solution (TBS: 0.005M Tris, pH 7.6[SIGMA, Munich, Germany]) for 5 min. renewing the buffer after half of the time.

AEC substrate

Peroxidase activity is revealed with the chromogen AEC (DAKO, Hamburg, Germany K3464) for 10 min. at room temperature. Nuclei of cells are subsequently counterstained in Mayer´s acid hematoxylin for 90 sec., rinsed under current tap water for 5 min. and transferred to distilled water.

Microscopic evaluation

In order to protect the sections, the slides are covered with plastic lids and fixed with Kaiser´s glycerol gelatin (MERCK, Darmstadt, Germany, cat. # 1.09242).

All breast tissue sections which reacted with a specific colouration which was more intense than the colouration of the background were evaluated as positive. The evaluation was effected under the microscope with a .. fold magnification. The reaction of tumour cells and extra cellular matrix plus stroma cells was evaluated manually and the intensity of the colouration was determined according to the following categories: - (no colouration) and + (colouration). Liver and kidney tissue sections were used as a positive material to demonstrate the presence of IDE in the evaluated samples.

Specificity testing of the polyclonal anti-IDE antibody (Rosner, Chicago, USA)

For determining and evaluating the specificity of the obtained rabbit anti-IDE antibodies raised against recombinant full-length human IDE, they were pre-incubated with the recombinant full-length human IDE (Data not shown). Therefore we applied different concentrations of the recombinant IDE and pre-incubated the rabbit polyclonal IDE-antibodies with it before applying them on kidney tissue samples. The

inhibition of the antibodies was not seen in the immunostaining of the kidney tissue samples. We can conclude that the immunostaining of the kidney tissue samples yielded negative.

4 Results

The present study investigated the expression pattern of the IDE protein in human malignancies vs. normal tissues. Therefore we examined by immunohistochemistry normal breast epithelium and breast cancer tissue samples for the presence of the insulin-degrading enzyme. For the immunohistochemical analysis of IDE two groups of antibodies were tested. While one group were rabbit polyclonal anti-IDE antibodies raised against recombinant full-length human IDE (Rosner, Chicago, USA), the other group were IDE antibodies directed against synthesized IDE-peptide p15 (YKEMKAVDAPRRHK) (Prof. M. Schmitt, Munich, Germany) from rabbit and from chicken (see Table 4). For the present study the POX detection method demonstrated the best staining results. More exactly the POX detection method was modified by pre-treating the tissue samples with pressure-cooking in citrate buffer for the antigen retrieval.

4.1 Testing of the polyclonal anti-IDE-peptide antibodies (Prof. M. Schmitt, Munich, Germany) (see Table 4)

Furthermore, polyclonal unpurified IDE-peptide antibodies (Prof. M. Schmitt, Munich, Germany) from rabbit of the following days elapsed since immunisation: 90, 120 and 160 were applied on kidney tissue samples. These antibodies were tested in different dilutions according to the respective protocols. IDE-peptide antibodies from the days 90 and 120 diluted in antibody diluent (1/100) showed an identical and nearly characteristic staining pattern in kidney tissue. Consequently, the staining is independent from the immunisation days. The cytoplasm of kidney epithelial cells was stained. There was no difference in the distribution of IDE immunoreactivity between proximal and distal tubules epithelial cells. But some tubules showed a stronger staining. Glomeruli were negative. The interstitial was also negative. A slightly positive reaction was detected in the smooth muscles of the blood vessels and in the endothelial cells of capillaries and of blood vessels. A significant difference in IDE expression was noticed between different rabbits (1 and 2). The staining of IDE-peptide antibodies from the day 160 was negative. To avoid unspecific reactions they were purified.

The purification of the IDE-peptide antibodies from the day 160 also showed a negative result in immunohistochemical analysis.

From chicken we produced polyclonal not-purified IDE- peptide antibodies of the following days: 90, 120 and 185. The immunostaining of the chicken anti-IDE antibodies was always negative independent of their grade of purification.

We can summarize that IDE antibodies directed against the IDE-peptide from rabbit and chicken (see Table 4) are not as useful as the polyclonal anti-IDE antibodies raised against recombinant full-length human IDE (Rosner, Chicago, USA).

Although IDE-peptide antibodies gained from the immunized rabbit on days 90 and 120 showed a nearly characteristic staining pattern for IDE, we did not apply them for the immunohistochemical analysis of the breast tissue samples because the staining was weaker and less specific compared to the immunostaining of liver and kidney tissue samples with the other antibody (Rosner, Chicago, USA).

Thus, these anti-IDE antibodies could be useful in the future but it will be necessary to further improve their specificity (e.g. through further purification). More exactly the amount of IDE-peptide antibodies in rabbit sera should be determined exactly to estimate the immunostaining results. In order to improve the staining results and to avoid unspecific reactions the purification of the IDE-peptide antibody sera should be revised. In addition we noticed a difference in the staining results of the different immunisation days. While the anti-IDE antibodies from rabbit of the immunisation days 90 and 120 showed a nearly characteristic staining pattern for IDE, the anti-IDE antibody of the immunisation day 160 stained negative in the not-purified form as well as in the purified form. Altogether it is worthwhile to work on the development of the anti-IDE peptide antibodies in the expectation that their specificity in comparison to the other antibodies will attribute to better staining results.

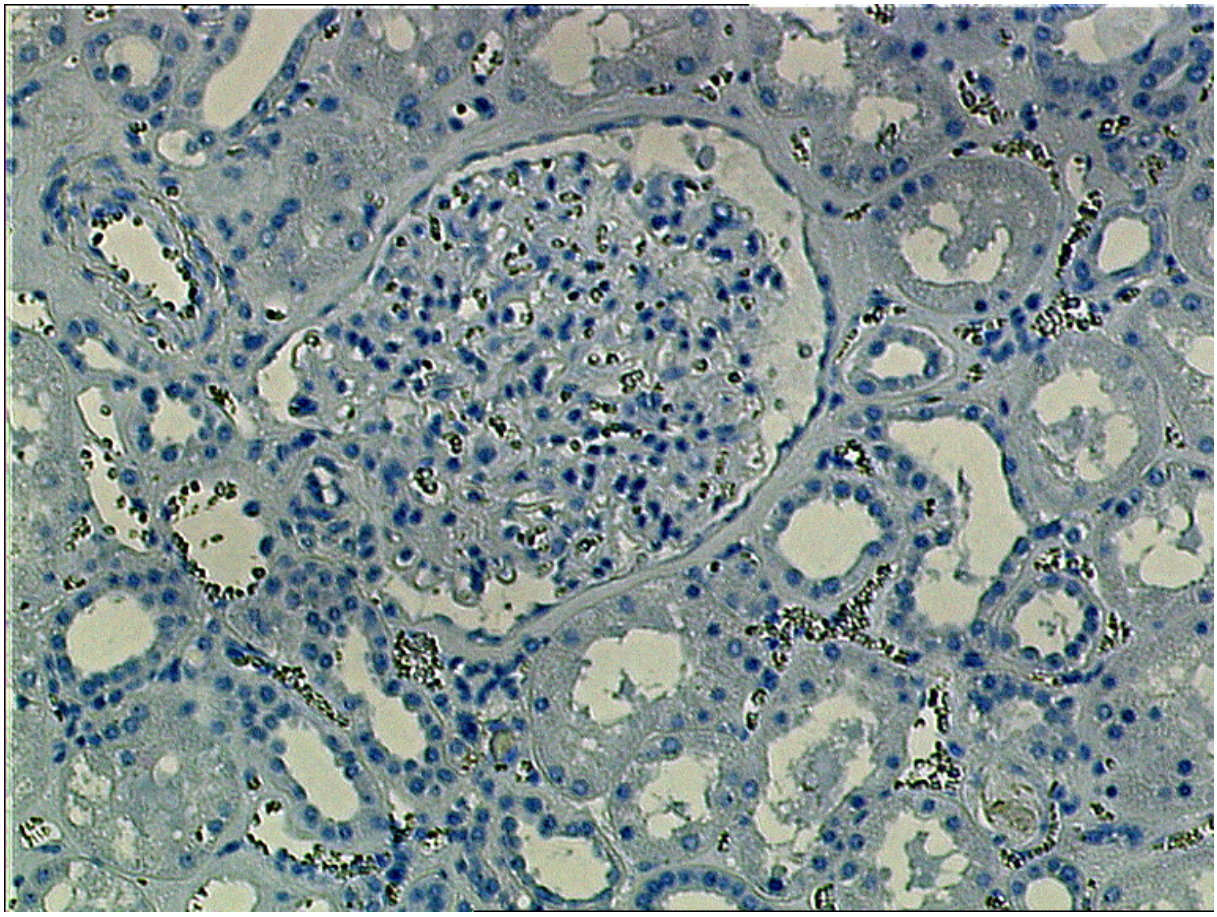


Figure 4: Normal kidney section serving as an IDE-negative control (ab to IDE peptide was left out) objective 10x

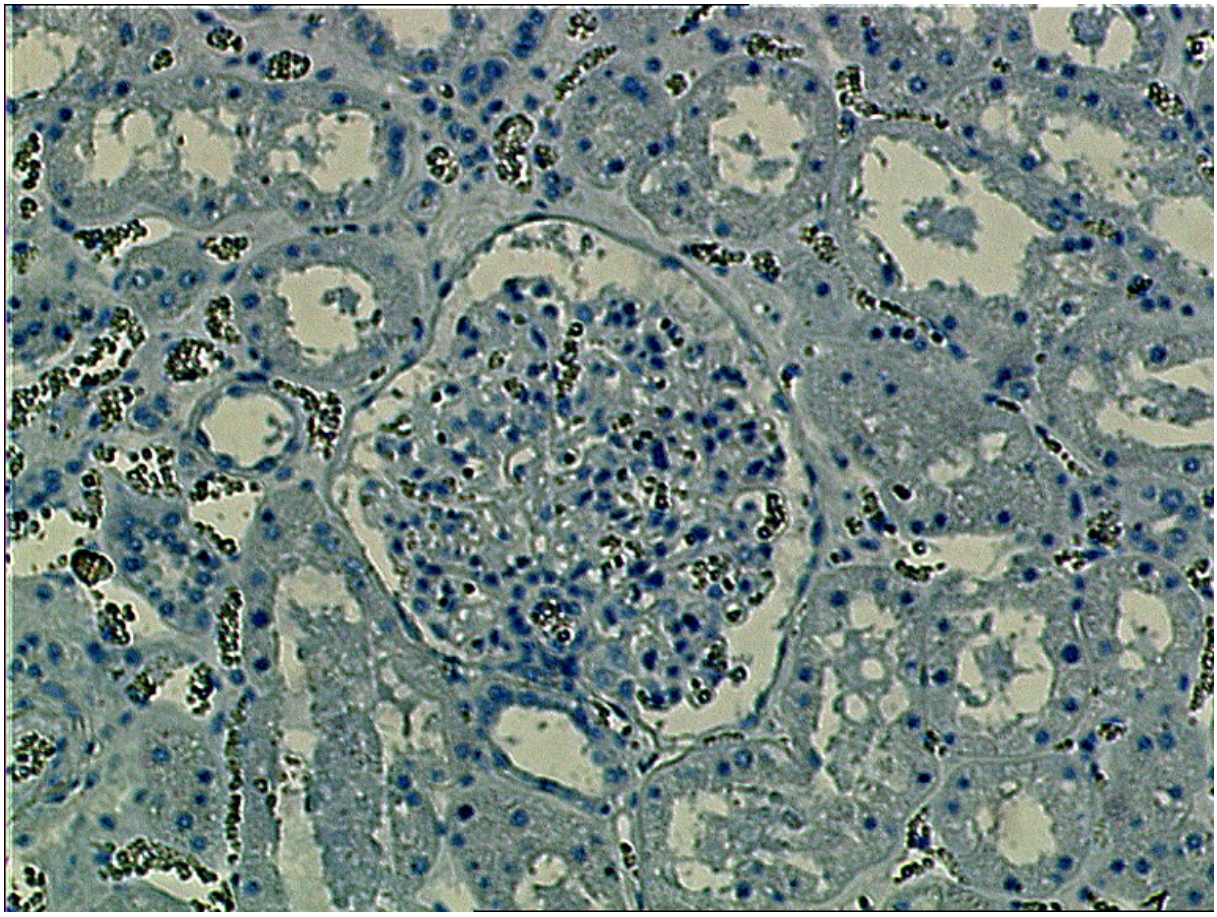


Figure 5: Normal kidney section serving as an IDE-negative control (ab to IDE peptide was left out) objective 10x

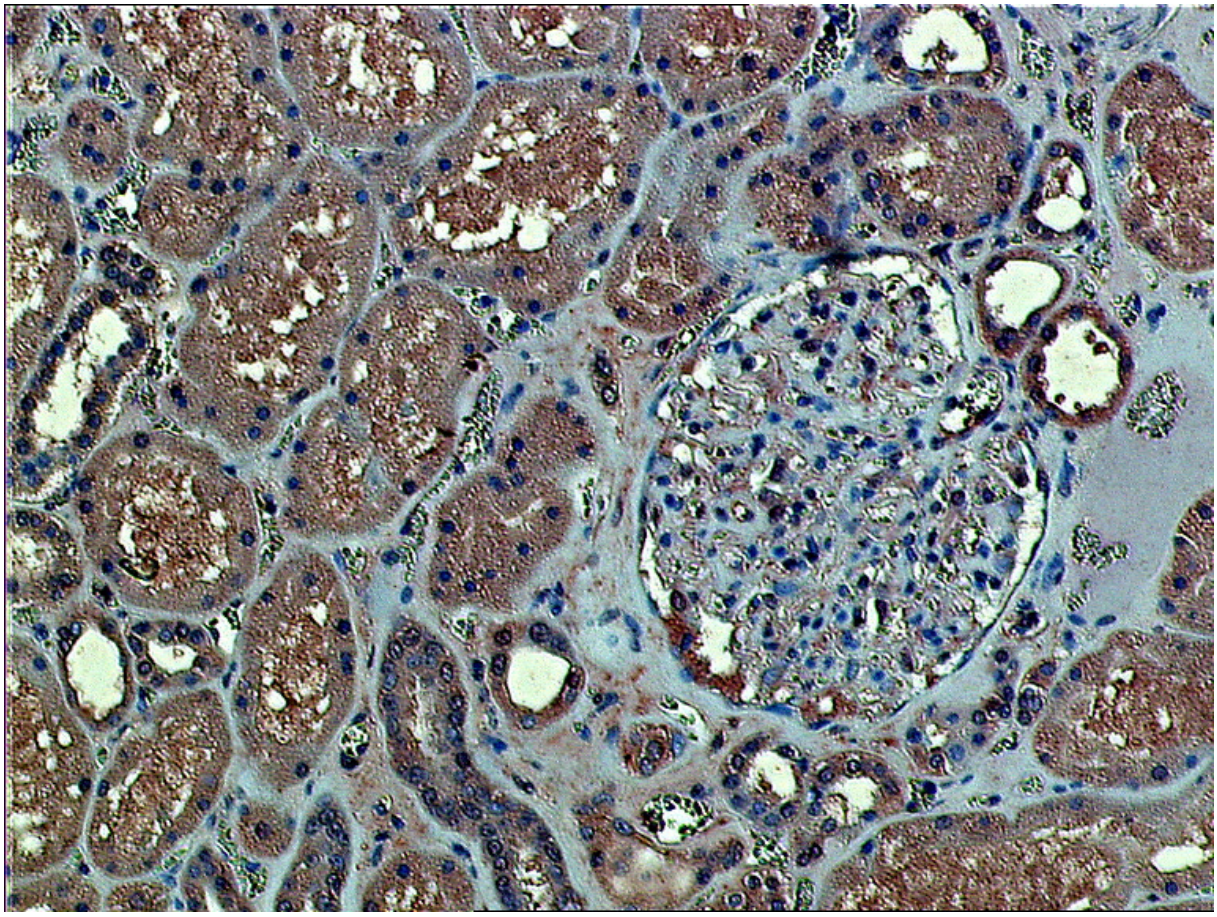


Figure 6: Normal kidney section, ab to IDE peptide, rabbit 1, 90d, 1:100, objective 10x

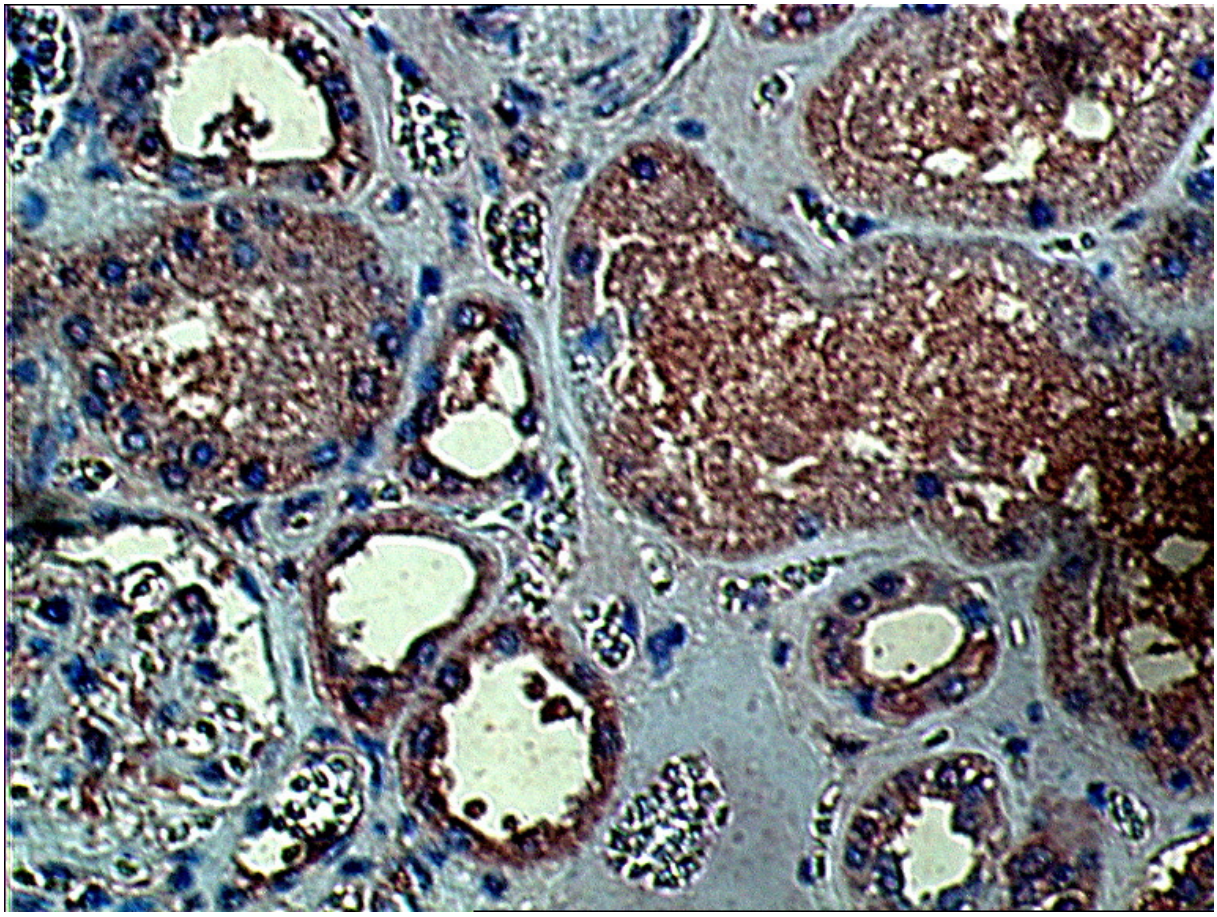


Figure 7: Normal kidney section, ab to IDE peptide, rabbit 1, 90d, 1:100, objective 20x

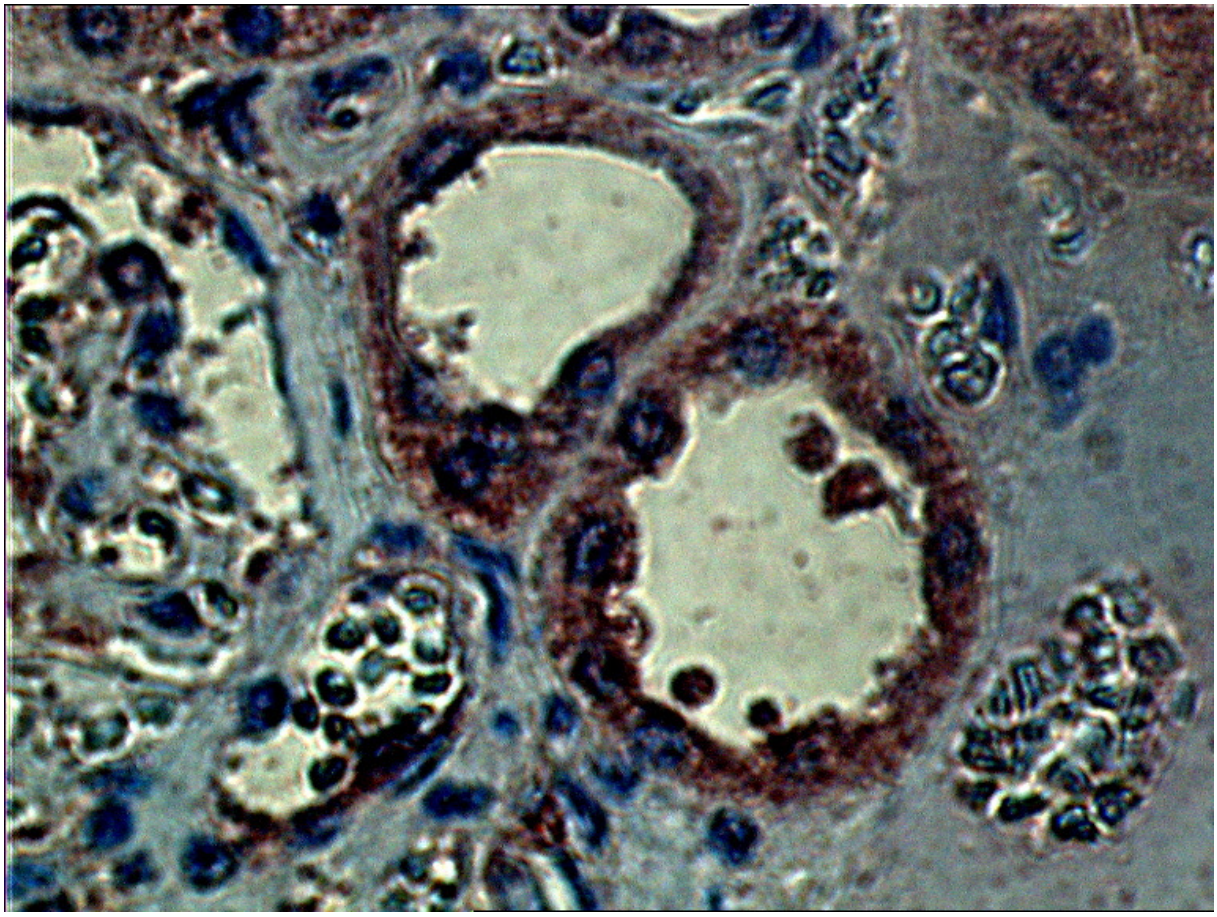


Figure 8: Normal kidney section, ab to IDE peptide, rabbit 1, 90d, 1:100, objective 40x

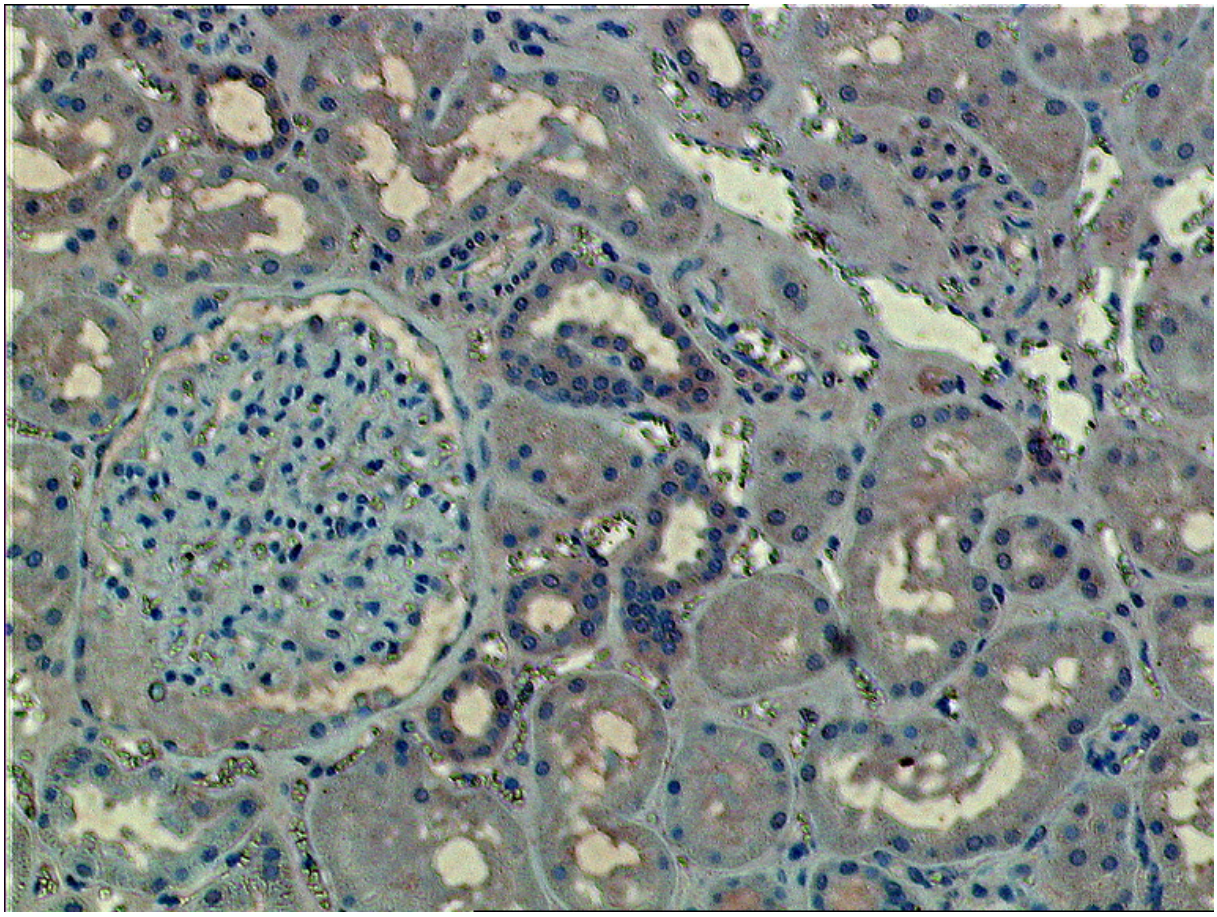


Figure 9: Normal kidney section, ab to IDE peptide, rabbit 2, 90d, 1:100, objective 10x

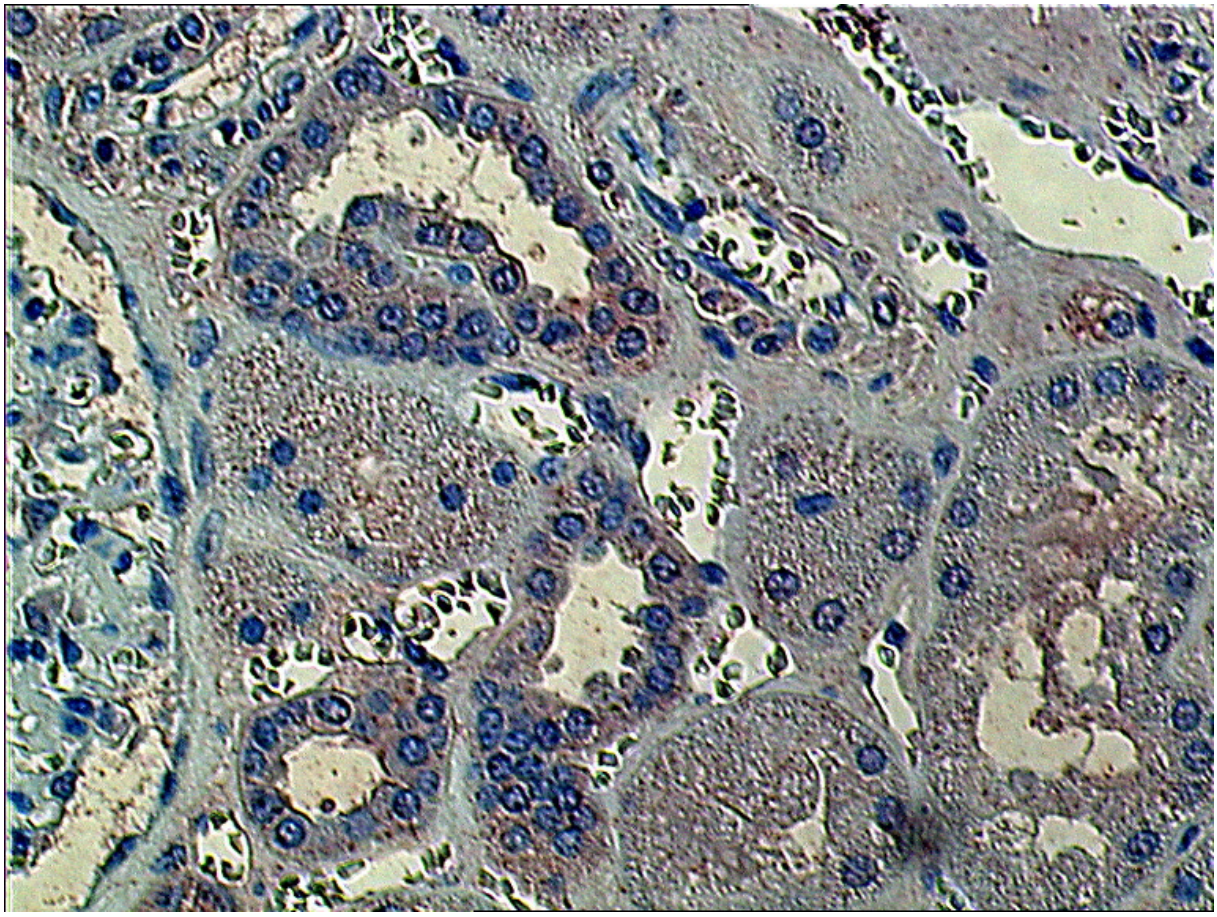


Figure 10: Normal kidney section, ab to IDE peptide, rabbit 2, 90d, 1:100, objective 20x

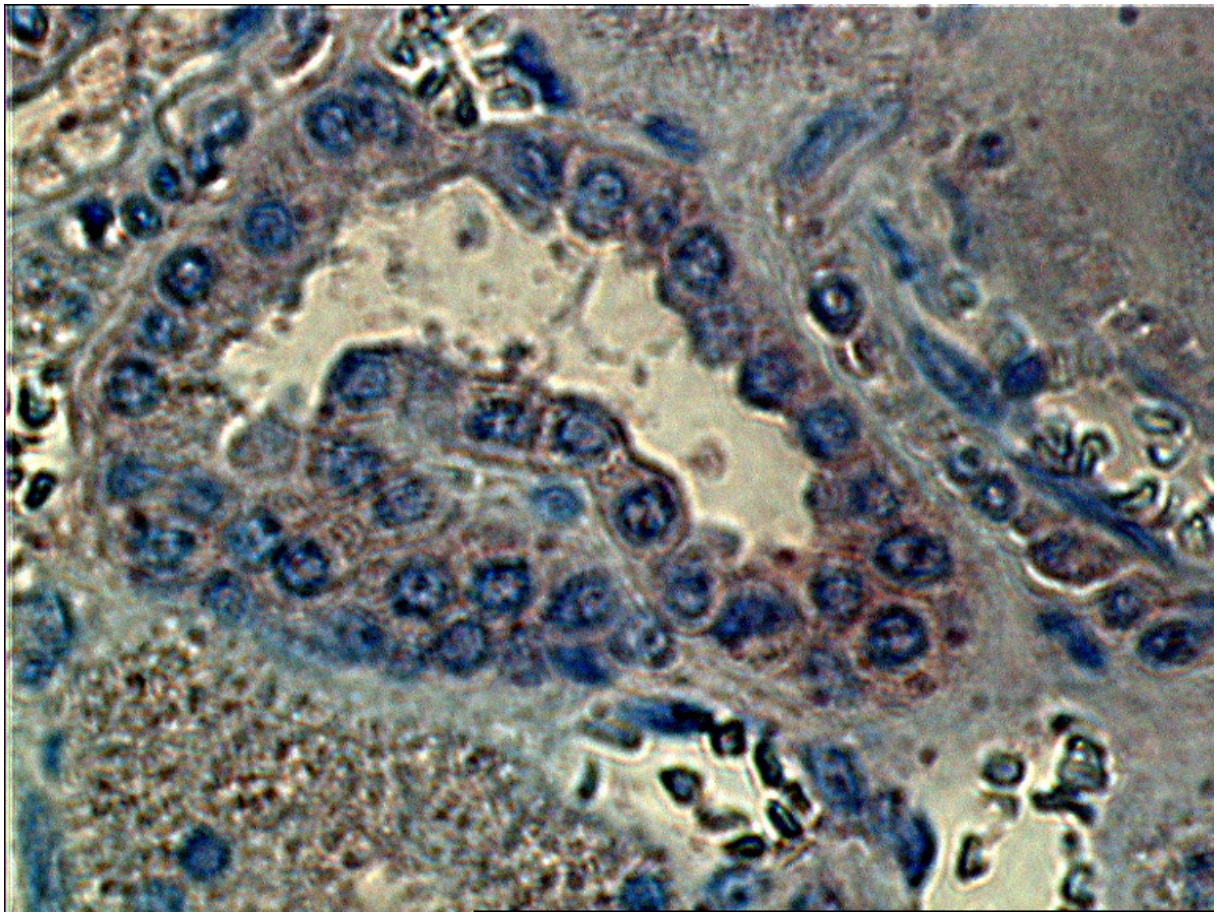


Figure 11: Normal kidney section, ab to IDE peptide, rabbit 2, 90d, 1:100, objective 40x

Table 4: Results obtained with Antibodies to IDE peptide (produced for Prof. M. Schmitt, Munich, by Pineda GmbH, Berlin)

Animal	Day	Purification	Dilution	Staining
Rabbit 1	90	Not-purified	1/100,1/500	Positive
	120	Not-purified	1/100,1/500	Positive
	160	Not-purified	1/650,1/800,1/1000	Negative
	160	purified	1/50,1/100	Negative
Rabbit 2	90	Not-purified	1/100,1/200,1/300, 1/500	Positive
	120	Not-purified	1/100,1/200,1/300,1/400,1/500	Positive
	160	Not-purified	1/650,1/800,1/1000	Negative
	160	purified	1/50,1/100	Negative
Chicken	90	not-purified	1/100,1/500	Negative
	120	Not-purified	1/50,1/100,1/200, 1/500	Negative
	185	Not-purified	1/100,1/500	Negative
	185	purified	1/50,1/100,1/200, 1/1000	Negative

Hufnagel, 2005

4.2 Testing of the polyclonal anti-IDE antibodies (Rosner, Chicago, USA)

Before the immunohistochemical staining was performed on breast tissues the procedure was established on formalin-fixed, paraffin-embedded sections of liver and kidney tissues and on multi-norm arrays which are known to show a characteristic staining pattern. The immunostaining of human liver with rabbit polyclonal anti-IDE antibodies raised against recombinant full-length human IDE (Rosner, Chicago, USA) diluted with antibody diluent (1/200) was homogeneous throughout central and portal vein regions. (Fig. 12) These results are not consistent with previous findings according to which portal regions show a stronger staining in contrast to the central regions (Akiyama et al, 1988). The immunostaining of human kidney also showed an identical and characteristic staining pattern, IDE-positive in the tubular epithelium and IDE-negative in the glomeruli (Fig. 14). Consequently it fulfilled all criteria to be applied for the immunostaining of normal breast epithelium and breast cancer tissue samples.

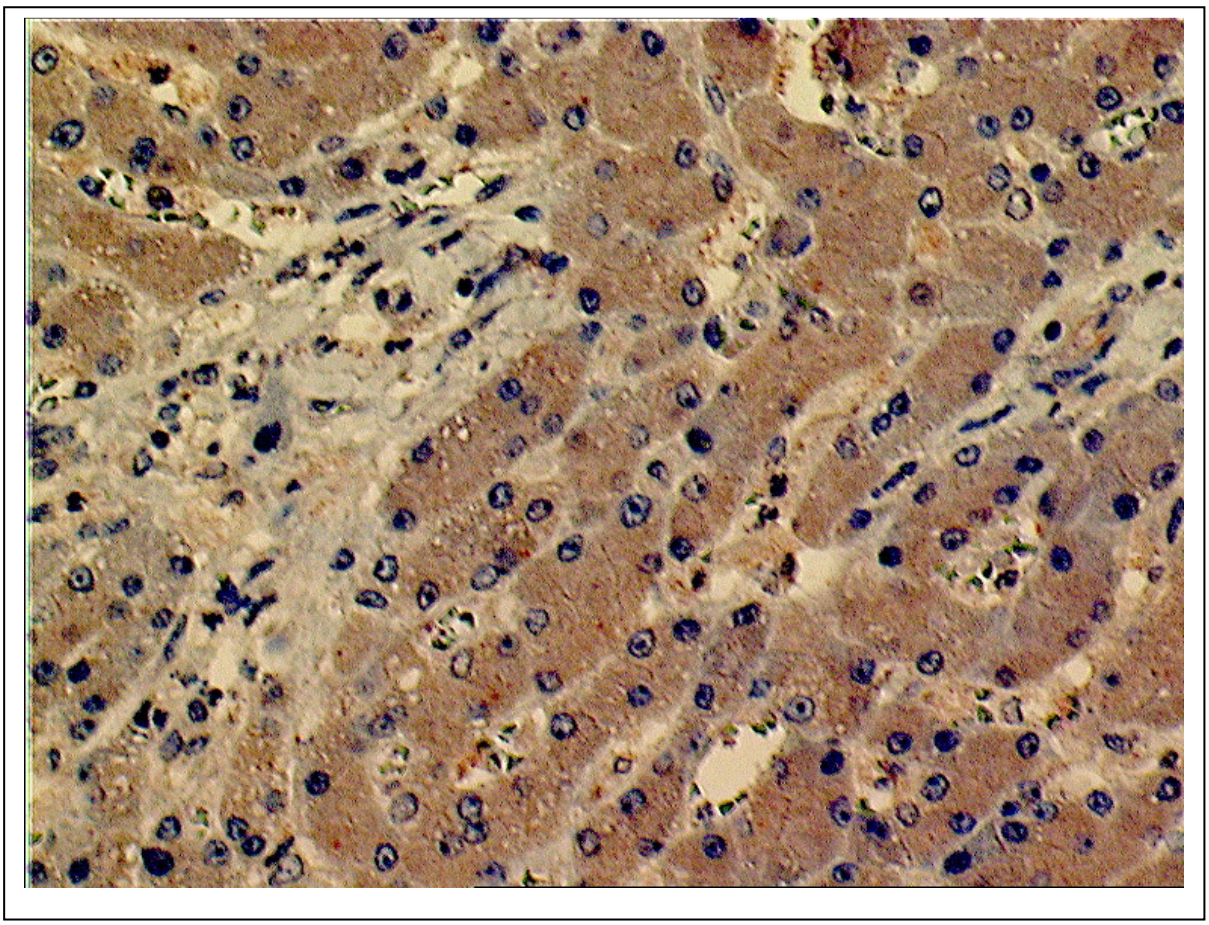


Figure 12: Normal liver section serving as an IDE-positive control, ab to full-length IDE, rabbit, 1: 200, objective 20x

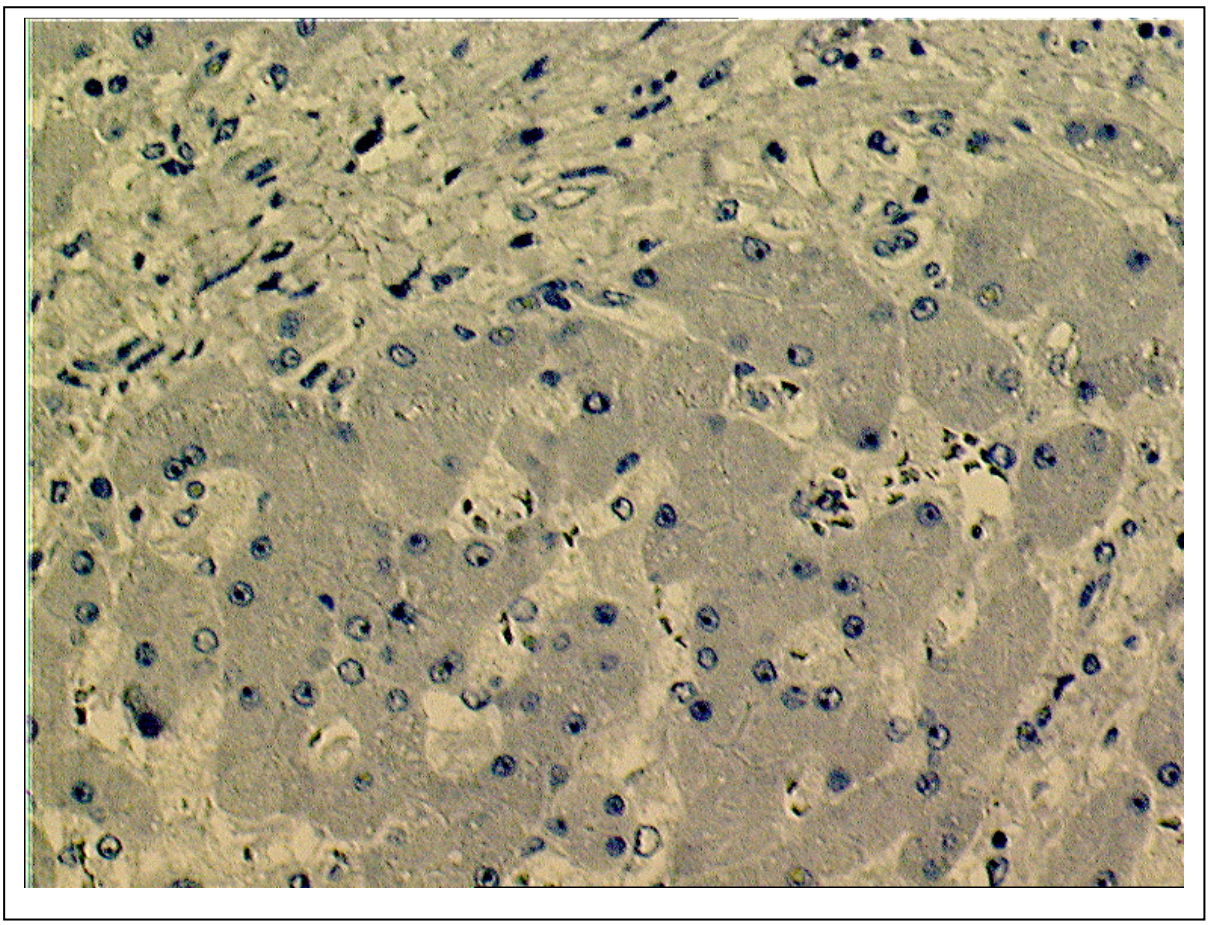


Figure 13: Normal liver section serving as an IDE-negative control (ab to full-length IDE was left out) objective 20x

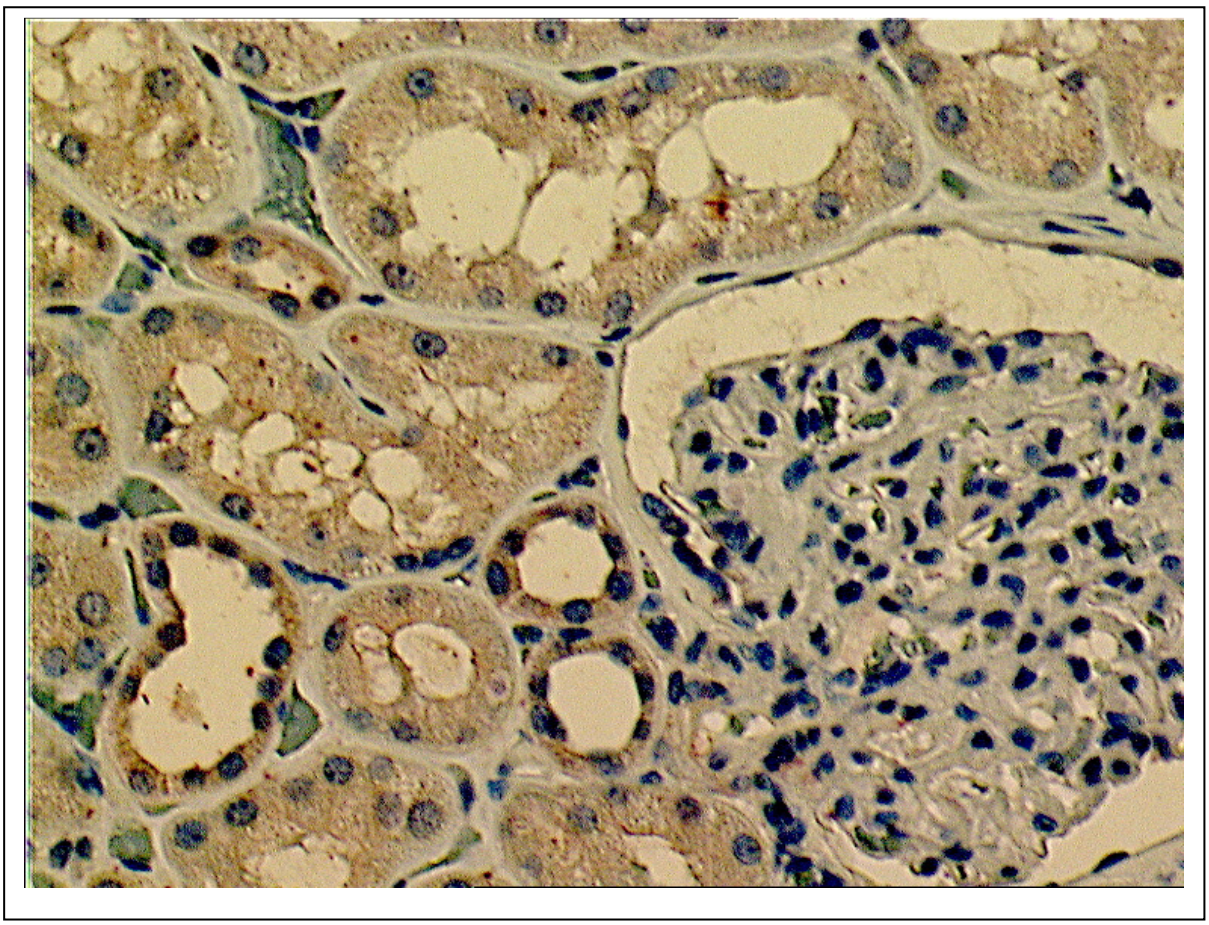


Figure 14: Normal kidney section serving as an IDE-positive control, ab to full-length IDE, rabbit, 1:200, objective 20x

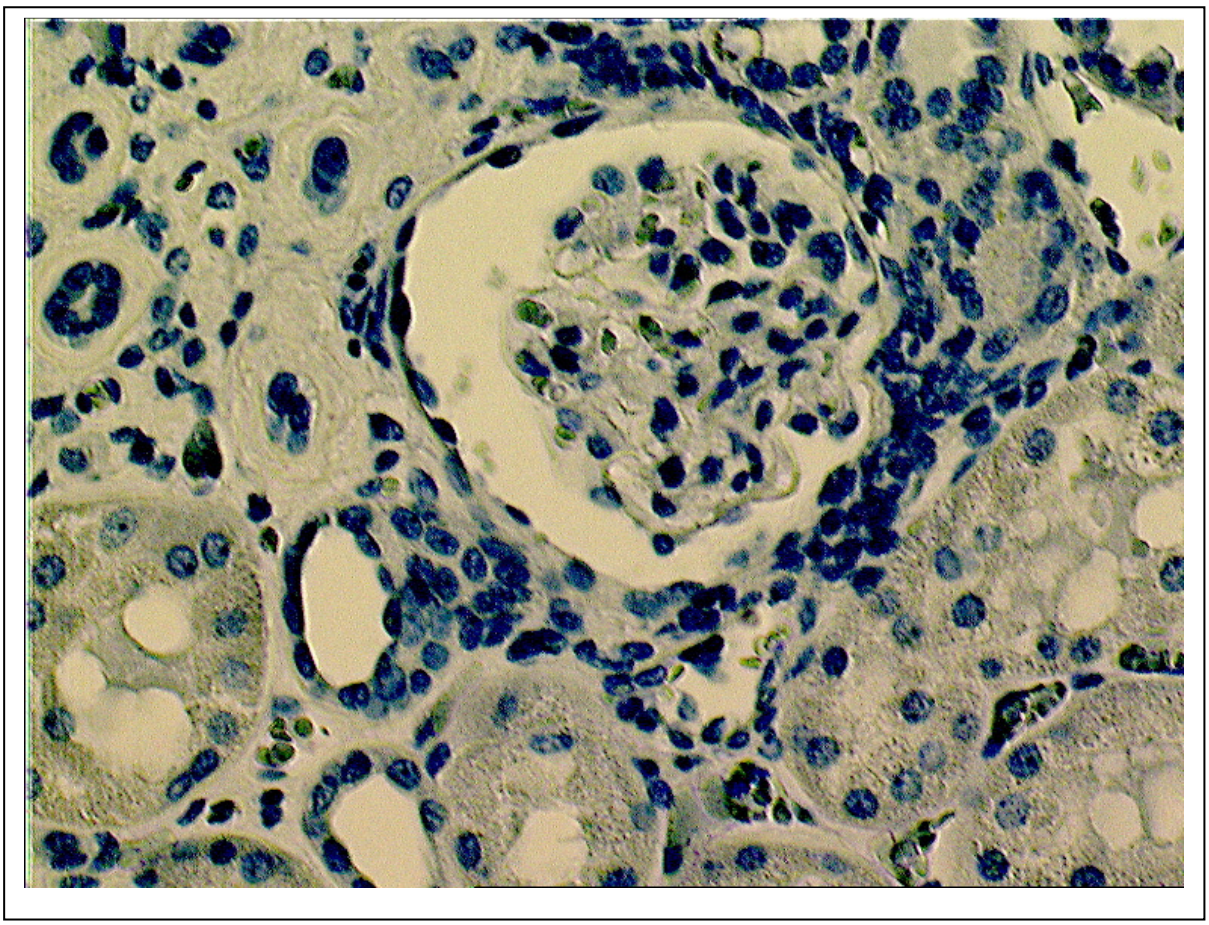


Figure 15: Normal kidney section serving as an IDE-negative control (ab to full-length IDE was left out) objective 20x

After testing these two groups of anti-IDE peptide antibodies, a rabbit polyclonal anti-IDE antibody raised against recombinant full-length human IDE (Rosner, Chicago, USA) was employed for the immunohistochemistry of human normal mammary gland and human breast carcinoma tissue samples.

4.3 IDE immunohistochemistry in normal human mammary glands

First we stained 24 samples of normal human breast tissues to study the expression pattern of IDE as compared to positive (Fig. 14) and negative (Fig. 15) staining controls. In all normal breast tissue samples IDE was only present in the cytoplasmic compartment and not in the nucleus (Fig. 16).The staining pattern was not uniform and the expression pattern of IDE was mostly focal, varying between individual cells (Fig. 16). More exactly 15 specimens (63%) demonstrated a heterogeneous staining whereby the epithelial cell layer contained stained and unstained cells. The epithelial cell layer of the remaining specimens were either mostly or entirely positive (5 specimens, i.e. 21%) or completely negative (4 specimens, i.e. 17%).

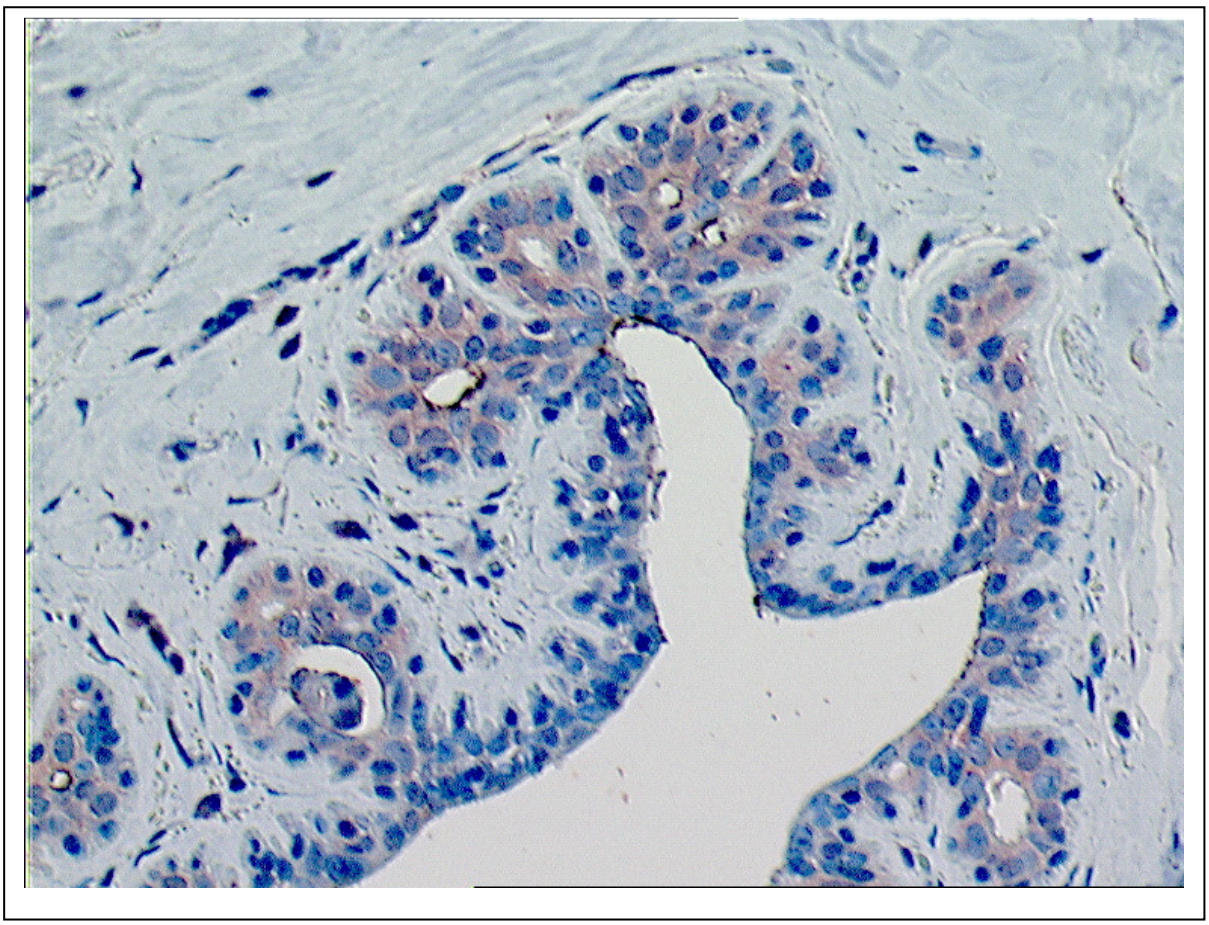


Figure 16: Normal human mammary gland with heterogeneous IDE expression (IDE-positive and -negative areas) ab to full-length IDE, rabbit, 1:200, objective 20x

4.4 IDE immunohistochemistry in human breast carcinomas

Then we stained a sequence of 23 cancerous human mammary gland tissues (Tab. 5). Most carcinomas (18 specimens, i.e. 78%) stained positive for (cytoplasmic, yet not nuclear) IDE (e.g. the grade 2 tumor #17 shown in Fig. 19 and the grade 3 tumor #7 shown in Fig. 17). The staining within the group of IDE-positive breast cancer tissues was present in all cells of the epithelial cell layer and had an homogenous pattern. The remaining 5 specimens (22%) were uniformly IDE-negative, specifically 3 of the 14 G3 tumors (21%), e.g. tumor #10 (Fig. 18), and 2 of the 8 G2 tumors (25%).

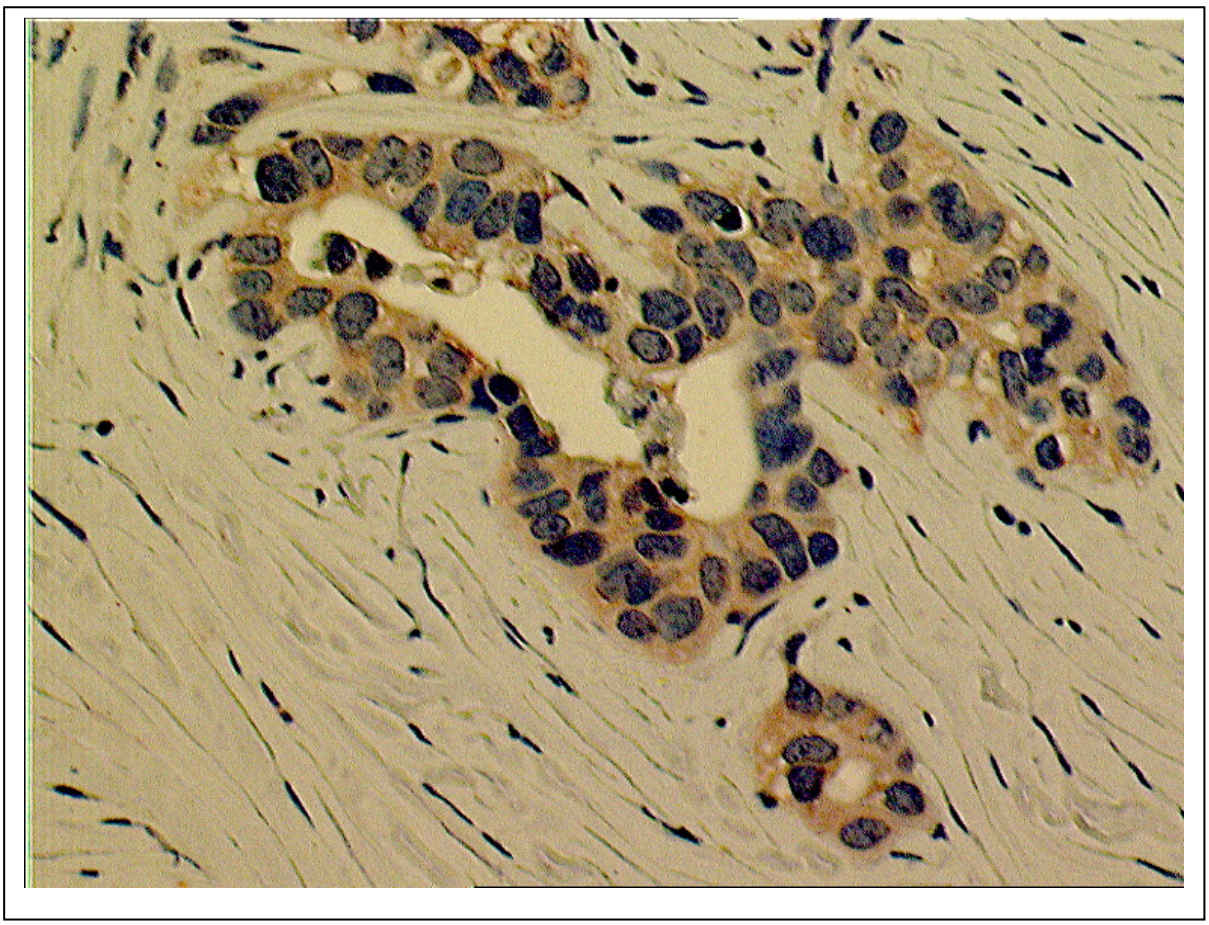


Figure 17: IDE-positive G3 breast carcinoma specimen #7, ab to full-length IDE, rabbit, 1:200, objective 20x

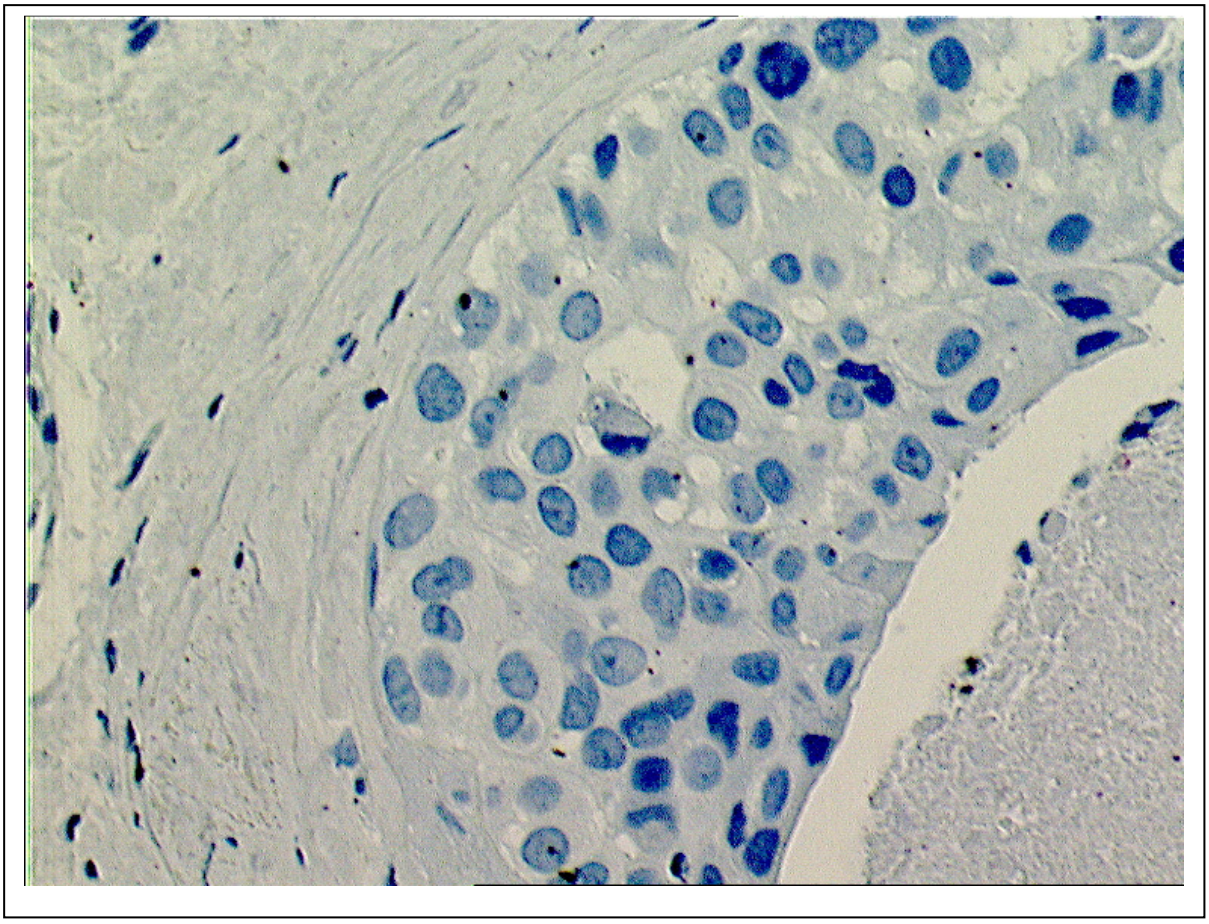


Figure 18: IDE-negative G3 breast carcinoma specimen #10, ab to full-length IDE, rabbit, 1:200, objective 20x

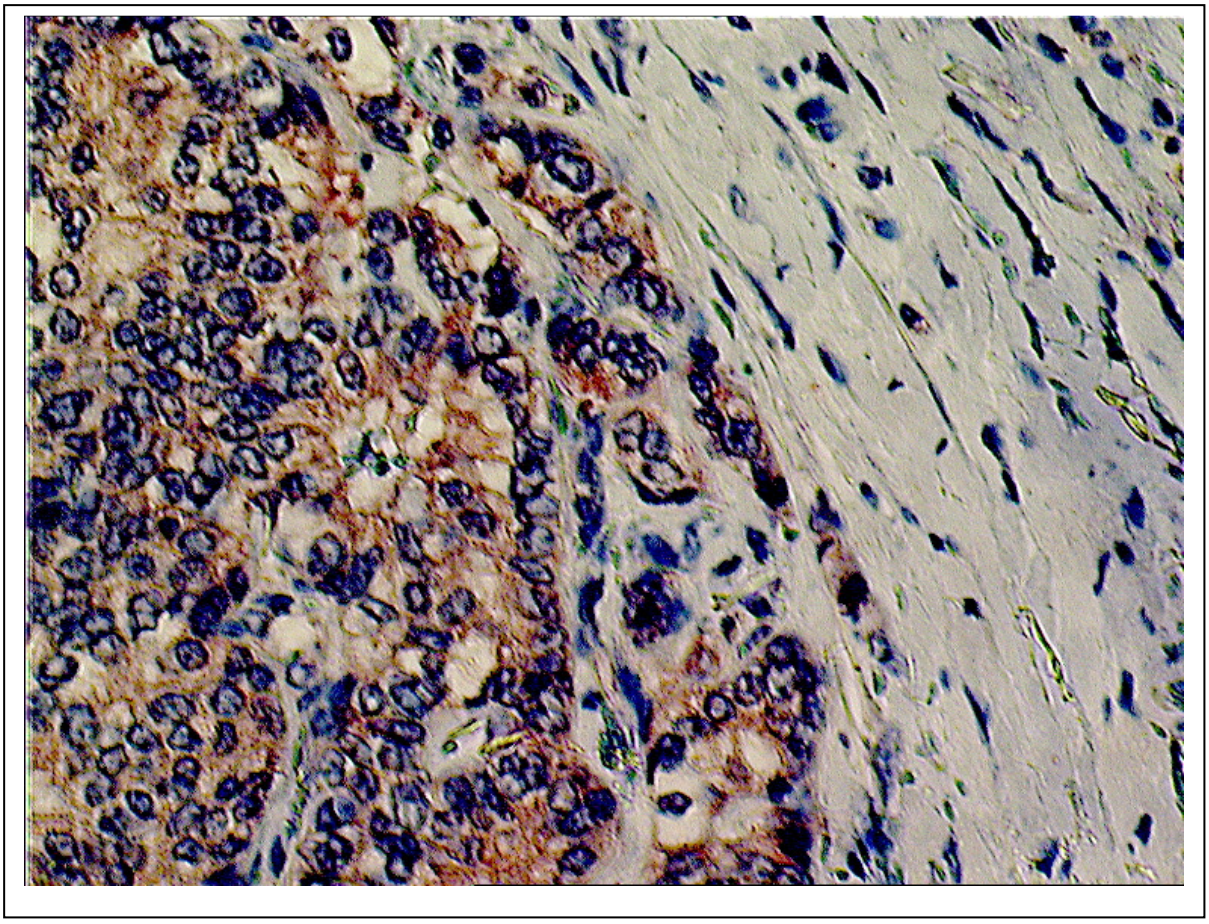


Figure 19: IDE-positive G2 breast carcinoma specimen #17, ab to full-length IDE, rabbit, 1:200, objective 20x

Table 5:

Breast Tumor #	Patient Age	Menopausal Status	Estrogen Receptor	Progesterone Receptor	HER2	T	N	M	G	IDE-IHC
1	44	peri	0/12	9/12	-	1c	1	0	3	-
2	65	post	12/12	6/12	-	2	0	0	3	+
3	58	post	0/12	0/12	-	2is	1	0	3	+
4	58	post	1/12	3/12	-	1c	0	0	3	+
5	64	post	2/12	0/12	-	2	1c	0	3	+
6	70	post	0/12	0/12	+	3	1	0	3	+
7	53	post	0/12	0/12	+	2	0	0	3	++
8	45	n.a.	0/12	0/12	+++	2	0	0	3	+
9	40	pre	0/12	0/12	-	2	0	0	3	-
10	66	post	0/12	0/12	-	1c	0	0	3	-
11	82	post	0/12	0/12	-	2b	1bi	0	3	++
12	47	n.a.	0/12	0/12	-	1c	1bi	x	3	++
13	45	pre	2/12	12/12	+++	1a	0	0	3	+
14	58	post	0/12	0/12	+	2	0	x	3	+
15	73	post	9/12	9/12	-	2	0	0	2	+
16	58	post	12/12	4/12	+	2	0	0	2	-
17	61	post	9/12	0/12	++	2	0	0	2	++
18	55	pre	12/12	12/12	-	1c	0	0	2	+
19	65	post	6/12	2/12	+	1c	0	0	2	-
20	68	post	12/12	12/12	-	1c	1a	0	2	+
21	71	post	9/12	0/12	-	1c	0	0	2	+
22	45	pre	12/12	9/12	+	1c	0	0	2	+
23	58	n.a.	0/12	0/12	-	2	0	0	1	+

modified from Radulescu *et al., International Journal of Oncology*, **30**, 73-80, 2007

5 Discussion

The present study has investigated by immunohistochemistry the expression pattern of the protein insulin-degrading enzyme (IDE) in a series of specimens of normal and malignant breast tissue samples. For the detection of the presence of IDE in breast tissue samples we first established an immunohistochemical protocol on normal liver and kidney tissue samples. Thereby we employed two types of antibodies: a rabbit polyclonal antibody raised against recombinant full-length human IDE (Prof. Rosner, Chicago USA) and IDE antibodies directed against the synthetic IDE peptide p15 (YKEMKAVDAPRRHK) (Prof. M. Schmitt, Munich, Germany) from rabbit and from chicken and analyzed their advantages and disadvantages.

The rabbit polyclonal anti-IDE antibody raised against the full-length human IDE has now been employed for the immunohistochemical staining of normal liver. We found a homogenous staining pattern throughout central and portal vein regions. By comparison, a heterogeneous staining of rat liver has been reported whereby portal regions showed a stronger staining than central regions (AKIYAMA et al, 1988).

Secondly IDE-antibodies raised against an IDE-peptide fragment were also tested on human kidney tissue samples in our laboratory. The antibody diluent served as negative control as above. By obtaining anti-IDE antibodies from different immunisation days (90, 120, 160, 185) and applying them in different dilutions we tried to find an antibody which showed a more characteristic staining pattern of IDE than the IDE antibodies from Rosner which were directed against the whole protein IDE. The most promising results with this antibody group were obtained by the rabbit IDE-peptide antibodies from the immunisation days 90 and 120 diluted in antibody diluent 1 to 100 which showed an identical and nearly characteristic staining pattern in kidney tissue. The cytoplasm of kidney epithelial cells stained as well as the proximal and distal tubules epithelial cells. Glomeruli and the interstitial were negative. A slightly positive reaction was detected in the smooth muscles of the blood vessels and in the endothelial cells of capillaries and of blood vessels. A significant difference in IDE expression was noticed between different rabbits. To avoid unspecific reactions we purified the antibodies. The staining of the human liver and kidney tissue samples

with the purified chicken polyclonal rabbit anti-IDE peptide antibodies was negative. In addition the quality of the staining with the IDE-peptide antibodies from rabbit of the immunisation days 90 and 120 was not comparable with the quality achieved by the use of the antibody against recombinant IDE (Rosner, Chicago). This led us to the conclusion that the purification process of the IDE-peptide antibodies has been insufficient and should be improved to diminish indistinct staining results.

Because of suboptimal staining results obtained with the anti-IDE peptide antibodies, we proceeded with the use of the rabbit polyclonal antibody against recombinant IDE (Rosner, Chicago, USA) which showed a characteristic staining pattern, for the immunohistochemical detection of IDE in human breast tissues and breast cancer tissues. Within this series of experiments, kidney tissue samples served as positive (Fig. 14) staining control because IDE is present in kidney epithelial cells. The antibody diluent was employed on kidney tissues as negative control (Fig. 15) as described above. Furthermore the POX detection method proved to be the optimal method for IDE detection modified by pre-treating the breast tissue samples by pressure-cooking in citrate buffer. Other techniques to achieve the best antigen retrieval like protease or vapour-cooking were not useful.

We are now the first to demonstrate that IDE is overexpressed in breast cancer specimens compared to normal human breast epithelium where only a small fraction was consistently IDE-positive (RADULESCU et al, 2007). After having established the staining method in normal human liver and kidney samples, we stained 24 samples of normal human breast tissues in order to examine the expression pattern of IDE (RADULESCU et al, 2007). To this end, we applied the antibody against recombinant IDE at a dilution of 1 to 200. The presence of the zinc dependent metalloendopeptidase IDE in the cytoplasmic compartment and its concomitant absence in the nucleus (Fig. 16-19) is consistent with other reports according to which IDE is a cytosolic enzyme (DUCKWORTH et al, 1998).

The staining pattern of the human normal breast tissues was not uniform and the expression pattern of IDE was mostly focal, varying between individual cells (Fig. 16). More exactly 15 specimens (63%) demonstrated a heterogeneous staining whereby the

epithelial cell layer contained stained and unstained cells. The epithelial cell layer of the remaining specimens was either mostly or entirely positive (5 specimens, i.e. 21%) or completely negative (4 specimens, i.e. 17%). We therefore conclude that the normal human breast is not a primary insulin target organ. Yet, the presence of IDE in some breast epithelial cells implies the involvement of IDE in physiological events like cell differentiation and protein metabolism in a fraction of these cells.

Consistent with our intention to compare the presence of the insulin-degrading enzyme in normal human breast epithelium vs. breast cancer tissues, a series of 23 cancerous human mammary gland tissues (Tab. 5) was stained with the same anti-full-length IDE antibody (RADULESCU et al, 2007). In contrast to the human mammary gland tissue, we detected IDE in most of the breast carcinomas (18 specimens, i.e. 78%). This enzyme was localized in the cytoplasmic (yet not nuclear) compartment (e.g. the grade 2 tumour #17 shown in Fig. 19 and the grade 3 tumour #7 shown in Fig. 17) in accordance with previous reports (DUCKWORTH et al, 1998). The staining within the group of IDE-positive breast cancer tissues was present in all cells of the epithelial cell layer and had a homogenous pattern. The remaining 5 specimens (22%) were uniformly IDE-negative, specifically 3 of the 14 G3 tumours (21%), e.g. tumour #10 (Fig. 18), and 2 of the 8 G2 tumors (25%). Considering that IDE-positive mammary neoplasias can be divided in different grades whereas the differentiation goes from the well-differentiated grade 1 tumours up to the poorly differentiated grade 3 tumours we could make an interesting assumption and underline the anti-cancer role of IDE. IDE is less expressed in advanced grade 3 breast tumours which imply that the loss of IDE is associated with a poorer prognosis than the moderately differentiated grade 2 and 1 tumours which contained functional IDE (Table 5).

Taken together, we have found that IDE is overexpressed (78%) in breast cancer specimens in comparison to normal breast epithelium (21%) when comparing the respective uniformly positive tissues with one another (RADULESCU et al, 2007).

Thus, immunoreactive IDE was detected in a characteristic staining pattern in both breast tissues taking into account their special tissue structure and metabolism. Yet, while IDE displayed a homogenous pattern in the epithelial cell layer in breast cancer

tissue, normal breast epithelium showed a distinct heterogeneous pattern containing stained and unstained cells (RADULESCU et al, 2007).

Considering that insulin is a major substrate for IDE and that it is present in higher quantities in cancerous cells than in normal cells as previously determined by RIA by CASTRO et al, we can explain the overexpression of IDE in breast cancer (78%) as follows. The observed upregulation of the insulin-degrading enzyme might have been a consequence of the increased intratumoural insulin levels. This upregulation of IDE could be interpreted as a cellular response to high insulin levels, as shown in a previous study (ZHAO et al, 2004).

Our results are consistent with previously reported findings on the expression pattern of other tumour suppressors such as p16 (DAI et al, 2000), p73 (GUAN et al, 2003), nm23 (KAPITANOVIc et al, 2005) and maspin (TERASHIMA et al, 2005) in malignant vs. normal tissues. Interestingly, the compensatory role which was attributed to IDE has also been discussed for the maspin (KIM et al, 2003) and CTCF (DOCQUIER et al, 2005) candidate.

These initial observations suggest that IDE itself is a putative tumour suppressor that prevents the nuclear translocation of insulin and thus this growth factor's subsequent binding and inactivation of RB (RADULESCU et al, 2007).

Moreover, our current data on IDE are consistent with results obtained on other proteases or peptidases for which a tumour suppressor role is being assumed (RAMIREZ-MONTAGUT et al, 2004; DAI et al, 2001; GOYAL et al, 1998).

Furthermore, we think that the putative RB-protective role of IDE is functionally equivalent to that of the tumour suppressor PTEN (RADULESCU et al, 2007). As such, PTEN keeps RB in an active state by abrogating the nuclear translocation of cyclin D which would be followed by cyclin D binding and inactivation of RB (RADU et al, 2003). So the subsequent upregulation of the IDE protein as described above is seen as a negative feedback mechanism degrading the growth-promoting factor insulin (RADULESCU et al, 2007).

For many years, insulin degradation has been viewed as not relevant for the regulation of insulin metabolism in connection with cancer progression. In previous studies there have been suggestions that insulin interaction with and degradation by IDE may play a more direct role in generating insulin effects. Abnormalities of the insulin-degrading enzyme at the genetic and the protein levels influence insulin clearance and degradation and are present in various pathological conditions. Recently, studies on the involvement of IDE in human disease have mainly been focused on the fact that Alzheimer (BERTRAM et al, 2000) and type two diabetes mellitus (FAHRAI-RAD et al, 2000; KARAMOHAMED et al, 2003) patients display abnormalities on the IDE gene locus. By contrast, the expression pattern and functional role of IDE in human cancer have not been studied as yet except for some reports on IDE in human cancer cell lines, e.g. in the Caco-2 colon carcinoma cell line (CHANG et al, 1997), that, however, focused on the metabolic aspects of insulin degradation.

Therefore, the aim of our present work was to analyze the role of IDE in (breast) malignancies in comparison to normal tissues and thus to test the initial working hypothesis of this study according to which IDE is a putative tumour suppressor (RADULESCU, unpublished observation, 1994). Our observation and detection of IDE in normal breast tissues and breast cancer specimens could have various consequences. On the one hand, IDE might play an important role as a tumour suppressor regulating the amount of the growth factor insulin. Since insulin and RB have been shown to physically interact with one another in the nuclei of human cancer cells leading to increased cancer cell proliferation (RADULESCU et al, 2000), insulin degradation by an overexpressed insulin-degrading enzyme could predict a favourable prognosis of the respective cancer disease. On the other hand, the lack of IDE identified in some of the breast tumours of our cohort could turn out to be an indicator of poor prognosis in patients with breast cancer since, owing to this lack of IDE, insulin may not have been degraded and thus could not have been prevented from inactivating RB and thus from increasing tumour cell proliferation (RADULESCU et al, 2007).

Future studies will certainly have to determine the validity of these initial promising findings by investigating a larger amount of specimens. It may turn out that IDE (intratumourally and/or in the serum) can serve as a prognostic marker for breast cancer progression. It is hoped that the potential prognostic factor IDE will allow a reliable prognosis on the development of the illness similar to established prognostic factors such as lymph node staging, uPA in lymph-node negative breast cancer (JÄNICKE et al, 2001), and the cell surface receptor HER-2 (PICCART-GEBHART et al, 2005). Prognostic factors have become increasingly important for the management of a risk adapted and individual therapy over the last years. Moreover, IDE should be further investigated as to its potential to serve as a predictive marker indicating the success or failure of therapies. Such a possibility appears likely not only in the light of the present findings, but also given the results of a previous study that has revealed that P-gp, a major protein mediating (anti-cancer) drug resistance, may bind and thereby inactivate IDE (RADULESCU et al, 1998).

6 Summary

Based on previous findings describing the known association of the growth factor insulin with mammary neoplasias, we have investigated the expression of the insulin-degrading enzyme (IDE) by immunohistochemistry in the present study. Therefore a series of specimens of normal human breast and a comparable amount of normal breast cancer tissue samples were examined for the presence of the insulin-degrading enzyme (IDE) a primarily cytoplasmic, zinc-dependent metalloprotease.

Consequently anti-IDE antibodies directed against the IDE-peptide and anti-IDE antibodies raised against the full-length human IDE were evaluated for immunohistochemistry on human tissue samples. While the last ones showed the characteristic staining pattern and were applied on breast tissue samples, the production and the application of the IDE-peptide antibodies should be revised and improved.

Our results demonstrate a significant difference in the expression pattern of IDE in normal breast tissue in comparison to breast cancer tissue. In the epithelial cell layer of 24 normal human breast specimens IDE is expressed mostly focal, varying between individual cells and only a small fraction of these specimens (around 20 %) was either entirely positive or negative, respectively.

By contrast, the vast majority (almost 80 %) of the investigated 23 breast cancer specimens was consistently IDE-positive whereby staining was observed in a homogeneous fashion in all of these cancer cells. These results suggest a compensatory upregulation of this protein during the transition from a normal to a malignant breast phenotype.

In this context the insulin-degrading enzyme could play a decisive role in the search for proteases as novel prognostic and predictive markers for human breast cancer. Taken together, these findings form a first experimental basis for future studies about the putative role of IDE as a tumour suppressor as well as the potential of IDE to serve as a clinical marker in cancer.

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