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HISTOPHOTOMETRIC QUANTIFICATION OF THE *FIELD EFFECT* AND THE *EXTENDED FIELD EFFECT* OF TUMORS

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Histophotometric investigations have been made on samples of human skin. Fresh frozen serial sections were fixed and stained for either reactive protein thiols (PSH_r) or total reactive protein sulphur (TRPS) using modifications of the DDD-Fast blue B-method. In addition, total protein thiols (PSH_t) were stained with the Mercurochromcyanide-method, and proteins were stained using a modified amido-black procedure. Significant differences were found between the different tumours investigated and normal tissue, and also between apparently normal tissue adjacent to the tumours and normal tissue from patients without tumour. To reveal such tumour-related changes of apparently normal tissue, termed the *field effect of tumours*, a double quotient had to be calculated from the PSH_r- and TRPS-values determined from both epithelium (epidermis) and connective tissue. In addition, abdominal skin was investigated from patients without tumour and patients with tumours of the female genital tract, liver or breast. With the aid of the double quotient procedure, highly significant differences were found between normal abdominal skin of patients without tumours versus similar samples taken from patients with tumours. The tumour-related changes found with abdominal skin distant from the tumours have been termed the *extended field effect* of tumours. These general tumour-related changes, independent of the size, state or degree of malignancy of the distant tumour, could be shown to be due to changes in abdominal dermis.

KEY WORDS: Cancer, free radicals, field effect, extended field effect.

INTRODUCTION

In preceding publications we have reported on the histophotometric quantification of reactive protein thiols (PSH_r), total protein thiols (PSH_t), total reactive protein sulphur (TRPS) and proteins of normal cervix tissue, and of different tumours of the human uterine cervix[1–5] as well as skin tumours and normal human skin.[6,7] Carcinoma-*in situ* and invasive carcinoma of the uterine cervix, as well as basal cell epithelioma and invasive squamous carcinoma of the skin, are characterized by a significant decrease in the protein staining intensity and by a relative increase in the thiol content of their proteins compared with normal epithelium. Also, in a similar manner, the connective tissue adjoining the lesions of both uterine cervix and skin exhibited a characteristic decrease in the protein staining intensity accompanied by an increase in the thiol content of the proteins.

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In our previous work we showed that apparently normal tissue adjacent to tumours of the cervix also changed in a characteristic fashion.[3-5] Although diminished slightly compared with changes in the tumour tissue itself, these tumour-associated changes of apparently normal tissue, which are termed the *field effect* of tumours, proved to be quite similar to the changes observed with the tumours themselves and their adjoining connective tissue. The question arose: are these tumour-associated changes restricted to the immediate neighbourhood of the tumours, or could tumour-associated changes also be observed fairly distant from the tumours?

The aim of this work was to search for extended tumour-associated changes and, if existent, to compare them with the changes observed in the neighbourhood of tumours. Since we had quantified the *field effect* of skin tumours, abdominal skin was chosen for the investigation of tumour-associated changes of normal tissue with no immediate spatial connection to malignant tumours.

MATERIAL AND METHODS

Fresh frozen serial sections (10 μm) were prepared from samples of skin obtained from normal healthy persons, as well as from basal cell epitheliomas and invasive squamous carcinomas of the skin. Samples from tumours containing a part of normal, morphologically unchanged tissue were taken also for the investigation of the *field effect*.

For the investigation of the *extended field effect* fresh frozen 10 μm serial sections were prepared from abdominal skin of patients suffering either from noninvasive or invasive tumours located fairly distant from the sampling site on the skin that was used for investigation. The patients under study either suffered from a disturbance of the female genital tract, like dysplasia (cervical intra-epithelial neoplasia, CIN 1–3), carcinoma *in situ* (CIN 3) or invasive carcinoma of the uterine cervix, carcinoma of the ovaries, corpus carcinoma, endometrial carcinoma (adenocarcinoma); or suffered from breast or hepatocellular carcinoma. In the case of breast cancer, apparently normal skin from the same breast was taken for our investigations.

The fresh frozen serial sections were fixed immediately after preparation with ether-ethanol (1:1) and subsequently stained either for reactive protein thiols (PSH_r), or for total protein thiols (PSH_t), or for total reactive protein sulphur (TRPS) including PSH_t and the so-called reactive (mixed) disulphides, or for proteins.

The proteins were demonstrated histochemically using a modified amido-black staining.[8] PSH_t were stained using the Mercurochrom-cyanide method.[9] Modifications of the DDD-Fast blue B-method were used for the histochemical demonstration of both PSH_r and TRPS.[10]

The mean absorbances ($E/\mu m^2$) of the stained sections were determined quantitatively using an UMSP-I (C. Zeiss, Oberkochen) with associated data processing and a rapid scanning table attachment. The histophotometric measurements were performed at 560 nm for PSH_r- and TRPS-staining, and 520 nm and 620 nm for PSH_t- and protein-staining, respectively. The conditions for the UMSP-I were: circular measuring diaphragm 10 $\mu m\ \phi$; iris diaphragm 20 $\mu m\ \phi$; step length and line distance of the meandric scanning 10 μm, respectively. The optics used were: Ultrafluar 32/0.40, projective 10. Representative areas of both morphologically intact unfolded epithelium (epidermis) and adjacent connective tissue were selected for the histophotometric determination of the mean absorbances. In general, 7 selected areas were investigated in each part of a tissue.

The double quotient: The result of histophotometric measurements are mean absorbances ($E/\mu m^2$) of selected parts of the tissue. These $E/\mu m^2$-values not only depend on the histochemical reaction that is studied but, in addition, are influenced strongly by both the thickness and the morphological character of the sections. Although all sections were cut carefully to be 10 μm thick there is always some variability from section to section, especially when sections have to be prepared over a long period from samples from different donors and using several different types of microtome. Also, the differing morphological character of distinct kinds of tissue is a well-known but unavoidable fact. Both differing thickness and morphology strongly influence the variation of the histophotometrically determined $E/\mu m^2$-values; in consequence, the histophotometry is characterized by a wide statistical distribution of the single measured values. The result is that small differences cannot be demonstrated significantly due to the relatively high values of the standard deviations. To be able to measure small differences or changes, we had to find a method that eliminated or reduced the inadequacies of histophotometry, i.e. variations in thickness and morphological character. The problem with thickness can be eliminated substantially by relating $E/\mu m^2$-values measured within epithelium (Ep) to those determined from the immediately adjacent stroma (St). Both parts, immediately adjacent within a given section, should have appreciably the same thickness. Therefore, the Ep:St-ratio, being largely independent of variations in thickness, eliminates the thickness problem. In a similar way the morphology-problem is solved by another ratio. For the histochemical demonstration of both PSH_r and TRPS two serial sections are needed. The morphological character of the tissue within two consecutive serial sections should be virtually identical. Thus, the relation of PSH_r determined within the epithelium to TRPS of epithelium is independent of the morphological character of that section. In consequence, the PSH_r:TRPS-ratio eliminates the morphology-problem. The combination of both relations to yield a double quotient, should eliminate both problems. The double quotient (Q PSH_r:TRPS) is the relation of the PSH_r:TRPS-ratio determined from epithelium to the PSH_r-:TRPS-ratio measured from the adjacent connective tissue.

TABLE I

The double quotient values (Q PSH_r:TRPS) obtained from measurements on samples of abdominal skin. *Abbreviations*: n-EP, normal abdominal epidermis from patients without tumour; SR, stratum reticulare (connective tissue); an-Ep (NIT), apparently normal abdominal epidermis from cases with noninvasive tumours; an-Ep (IT), apparently normal abdominal epidermis from cases with invasive tumours; n, number of cases; mv, mean value; SD, standard deviation

Tissue	n	Q PSH_r:TRPS	
		mv	SD
1) n-Ep + SR (normal)	37	1.9	0.3
2) an-Ep + SR (NIT)	43	1.1	0.2
3) an-Ep + SR (IT)	28	1.1	0.2

t-test:
2) vs 1) $p < 0.001$
3) vs 1) $p < 0.001$

RESULTS AND DISCUSSION

Samples of abdominal skin from 37 normal cases have been investigated. Normal cases, in this context, include patients without any tumour but either suffering from chronic cervicitis or from leiomyoma uteri. Samples from patients that had pathological abnormalities of either the female genital tract, or of liver or breast, have been divided into two groups: (i) abdominal skin from 43 patients with pre-malignant lesions or non-invasive tumours (NIT), including dysplasias of the uterine cervix (CIN 1–3); and (ii) skin samples form 28 patients with invasive tumours (IT). Table I lists the double quotient values (Q PSH_r:TRPS) found with samples of abdominal skin. A highly significant difference exists ($P < 0.001$) between the mean value (1.9) of the normal group and both of the mean values of the cases with pre-malignant lesions and non-invasive tumours (1.1) and with those with invasive tumours (1.1). These highly significant differences of the double quotient values indicate the existence of an *extended field effect* in the samples studied.

The distributions of the single double quotient values determined with abdominal skin are illustrated in Figure 1. An overlap of less than 5% of the double quotient values determined with normal cases versus those obtained with samples taken from

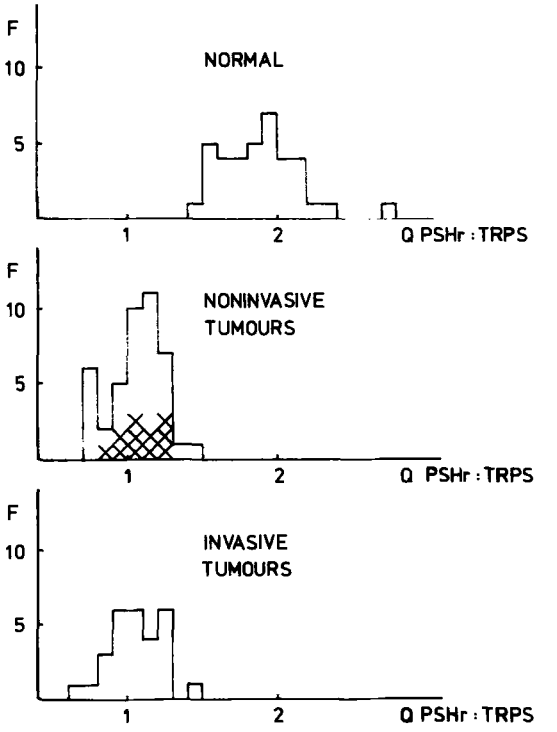

FIGURE 1 The *extended field effect* of tumours. Distribution of the double quotient values calculated from measurements on samples of abdominal skin obtained from patients without tumour (NORMAL), and from patients suffering either from noninvasive lesions or tumours (values relating to dysplasias, CIN 1–3, are indicated by X), and from invasive tumours. Ordinate, absolute frequency (F); abscissa, double-quotient values.

patients with pre-malignant lesions, NIT, and IT has been found. Thus, at first sight, the *extended field effect* (EFE), if demonstrated with the double quotient (Q PSH_r:TRPS), could be used in principle for tumour detection: this would be quite a new kind of detection since there would be no need to find precisely the tumour location and, in addition, the value found is clearly also independent of the size of the tumour. However, CIN 1–3 as well as pre-malignant lesions investigated exhibited the same *extended field effect* as solid invasive tumours so that the changes found are not specific for invasive tumours. Moreoever, in practice, the use of the double-quotient-method is very restricted and is unsuited to routine use. It must be mentioned again that this method needs first of all two well prepared morphologically equivalent sections for the staining of both PSH_r and TRPS and furthermore a 14-days staining for the histochemical demonstration of TRPS. The actual quantitative histophotometry of PSH_r and TRPS, however, can be performed easily and is not time consuming.

What are the biochemical changes in skin that underlie the changes in the double ratio observed between abdominal skin of *healthy* patients and patients suffering from

TABLE II

Histophotometrically determined mean absorbances ($E/\mu m^2$) of reactive protein thiols (PSH_r), total reactive protein sulphur (TRPS), proteins and total protein thiols (PSH_t) in the epidermis of samples of abdominal skin from normal patients and those with noninvasive tumours including CIN 1–3, dysplasias (CIN 1–3) or invasive tumours

Group		PSH_r		TRPS		Protein		PSH_t	
	n	mv	SD	mv	SD	mv	SD	mv	SD
1) Normal	37	0.87	0.28	1.96	0.82	0.74	0.52	1.03	0.30
2) Noninvasive tumours	43	0.81	0.31	1.86	0.76	0.71	0.53	0.99	0.35
2a) Dysplasias	11	0.79	0.30	1.77	0.40	0.63	0.59	0.96	0.25
3) Invasive tumours	28	0.77	0.36	2.09	1.09	0.68	0.50	0.99	0.32
t-test:									
1) vs 2)		n.s.		n.s.		n.s.		n.s.	
1) vs 3)		n.s.		n.s.		n.s.		n.s.	

TABLE III

Histophotometrically determined mean absorbances ($E/\mu m^2$) of PSH_r, TRPS, proteins and PSH_t in the dermis of samples of abdominal skin obtained from normal patients and from patients with noninvasive tumours, including CIN 1–3, dysplasias (CIN 1–3), or invasive tumours

Group		PSH_r		TRPS		Protein		PSH_t	
	n	mv	SD	mv	SD	mv	SD	mv	SD
1) Normal	37	0.19	0.07	0.80	0.37	0.32	0.37	0.29	0.09
2) Noninvasive tumours	43	0.24	0.12	0.56	0.27	0.26	0.30	0.27	0.10
2a) CIN	11	0.23	0.13	0.53	0.19	0.27	0.40	0.26	0.07
3) Invasive tumours	28	0.22	0.12	0.60	0.25	0.28	0.36	0.26	0.07
t-test:									
1) vs 2)		$P < 0.05$		$P < 0.01$		n.s.		n.s.	
1) vs 3)		n.s.		$P < 0.05$		n.s.		n.s.	

pre-malignant lesions and malignant tumours? What is the location of these changes observed with the parameters PSH_r, TRPS, PSH_t and proteins? Is the EFE observed by using the double quotient method due to changes of the epidermis, or of the dermis, or both? The data listed in Table II clearly indicate that the epidermis of abdominal skin is not the location of the EFE. Tumour-associated changes distant from the tumours are not manifested in changes of the epidermis. This seems to be an important finding especially in respect to a possible view that the EFE might be caused by a direct influence by tumour derived factors, e.g. (epidermal) growth factors. Table III clearly demonstrates the situations where tumour-associated changes are located fairly distant from the primary tumour, and the type of change involved: the change is primarily in the dermis. The most conspicuous change found between dermis of the normal group and dermis of abdominal skin of patients suffering either from NIT or from IT is a significant decrease in the TRPS-staining intensity. Together with a slight increase of PSH_r and unchanged PSH_t this should indicate, first of all, a change of the thiol:disulphide ratio of dermal proteins characterizing the EFE of malignant tumours. It should be noted also that there is no significant decrease in the protein-staining intensity.

In this present study the EFE has been demonstrated with samples of abdominal skin. Thus, to study possible substantial differences between the FE and the EFE we have compared skin with skin. When changes were studied in the double ratio in samples obtained from apparently normal skin *adjacent* to skin tumours, it was found that these changes were largely independent of the type of the neighbouring tumour (Table IV); the distribution of individual values showed a big overlap with the double quotient values from normal skin (Figure 2). However, there are problems in comparing the *absolute* measurements listed in Tables II and III for the EFE with those listed in Table V and VI for the FE. Why are the values of Tables II and III much higher than those of Tables V and VI? The reason is that the values listed in Tables II and III have been obtained from abdominal skin samples from patients living in Thailand and the values listed in Tables V and VI are from Austrian patients. The stronger pigmentation in general of Thai skin leads to a considerably higher background staining, especially if stained with the DDD-Fast blue B-method. However, quan-

TABLE IV

The *field effect* of skin tumours. *Abbreviations*: n-Ep + SR, double quotient values of epidermis and dermis of normal skin from patients without tumours; an-Ep + SR, (BCE; ICS), double quotient values of normal epidermis and dermis of apparently normal skin adjacent to basal cell carcinomas and invasive squamous carcinomas of human skin, respectively

Tissue		Q PSH_r:TRPS	
	n	mv	SD
1) n-Ep + SR (normal)	33	2.0	0.3
2) an-Ep + SR (BCE)	66	1.3	0.5
3) an-Ep + SR (ICS)	16	1.4	0.3

t-test:
2) vs 1) $P < 0.001$
3) vs 1) $P < 0.01$

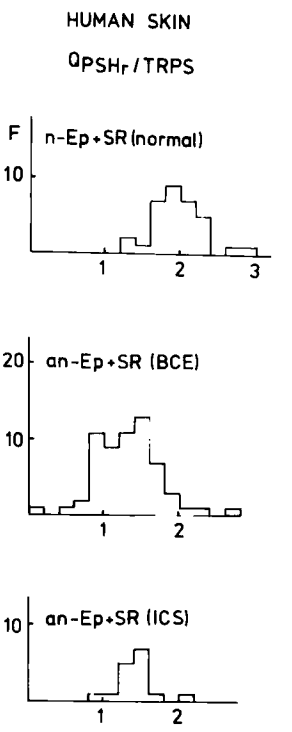

FIGURE 2 The *field effect* of tumours. The distribution of the double quotient values obtained from samples of normal human skin either of healthy donors (normal) or of patients suffering from an adjacent lesion or tumour (e.g. CIN or invasive carcinoma of uterine cervix (ICC); basal cell eipthelioma (BCE) or invasive squamous carcinoma of skin (ICS)).

titative differences between FE and EFE are quite independent of the differences in absolute values.

Table V lists the values obtained from samples of epidermis of normal skin from healthy patients and of skin adjoining to basal cell epitheliomas (BCE) or invasive

TABLE V

Histophotometrically determined mean absorbances ($E/\mu m^2$) of PSH_r, TRPS, proteins and PSH_t of human epidermis. Normal, BCE, ICS-epidermal $E/\mu m^2$-values of normal skin from patients without tumour, and of apparently normal skin adjacent to basal cell carcinomas and invasive squamous carcinomas of skin, respectively

Group		PSH_r		TRPS		Protein		PSH_t	
	n	mv	SD	mv	SD	mv	SD	mv	SD
1) Normal	32	0.37	0.12	0.82	0.23	0.59	0.35	0.86	0.17
2) BCE	66	0.31	0.09	0.82	0.21	0.49	0.23	0.86	0.26
3) ICS	16	0.36	0.10	0.88	0.19	0.50	0.31	1.02	0.31
t-test:									
1) vs 2)		$P < 0.01$		n.s.		n.s.		n.s.	
1) vs 3)		n.s.		n.s.		n.s.		$P < 0.05$	

TABLE VI

Histophotometrically determined mean absorbances (E/μm^2) of PSH_r, TRPS, proteins and PSH_t of human dermis. Normal, BCE, ICS-dermal E/μm^2-values of normal skin from patients without tumour, and of apparently normal skin adjacent to basal cell carcinomas and invasive squamous carcinomas of skin, respectively

Group		PSH_r		TRPS		Protein		PSH_t	
	n	mv	SD	mv	SD	mv	SD	mv	SD
1) Normal	32	0.09	0.05	0.36	0.18	0.20	0.15	0.31	0.08
2) BCE	66	0.08	0.03	0.28	0.10	0.13	0.09	0.28	0.08
3) ICS	16	0.10	0.03	0.35	0.11	0.14	0.11	0.33	0.15
t-test:									
1) vs 2)		n.s.		$P < 0.01$		$P < 0.01$		n.s.	
1) vs 3)		n.s.		n.s.		n.s.		n.s.	

squamous carcinomas of skin (ICS). Statistical analysis indicated only two significant differences: a significant decrease of epidermal PSH_r near BCE's and a significant increase of PSH_t near ICS. Although not significant, the 15% decrease of the staining intensities of epidermal proteins should also be noted.

The values for dermis listed in Table VI reveal two remarkable changes: (i) the significant decrease of dermal TRPS in the neighbourhood of BCE's, (ii) the significant decrease of the staining intensity of dermal protein next to BCE's, which to a similar extent could be observed in the neighbourhood of ICS's too. Only the TRPS values were decreased within the dermis of abdominal skin samples taken at a distance from tumours. This decrease of dermal TRPS, possibly characteristic for a general tumour associated change, could be seen only in the neighbourhood of BCE's, tumours of relatively low malignancy. Within dermis adjacent to invasive tumours this decrease of TRPS could no longer be detected. On the contrary, a relative increase occurred. In addition, dermis adjoining both BCE's or ICS's showed a characteristic decrease of the protein staining intensity.

It is clear from the studies reported here that the FE and the EFE are biological phenomena of considerable importance, and warranting much further investigation. Initially suggested by esr-studies of human cervix,[11,12] the FE was clearly established by measurements of rective protein thiols in human cervix.[5] The present study has widened the relevance of the FE to other locations and has raised a number of important new questions that will stimulate more studies.

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