Parameters Related to Oxygen Free Radicals in Human Skin: A Study Comparing Healthy Epidermis and Skin Cancer Tissue

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In vitro* studies with tumor cells have demonstrated that oxygen free radicals are involved in the development of skin cancers and that variations in the body's defense mechanisms can modify the course of the disease. To assess the validity of this hypothesis in spontaneous tumors, we determined glutathione S-transferase, superoxide dismutase, reduced and oxidized glutathione, and thiobarbituric acid reactive substances in healthy whole skin (n = 95), dermis (n = 73), and epidermis (n = 69). The values were compared with those obtained in three types of skin cancer: basal cell carcinoma (n = 16), squamous cell carcinoma (n = 6), and melanoma (n = 33). In healthy skin, glutathione S-transferase, superoxide dismutase, reduced glutathione, and oxidized glutathione were higher in epidermis than in dermis, whereas thiobarbituric acid reactive substances were higher in dermis than in epidermis; whole skin had intermediate values. These results suggest that there is an induction of some anti-oxygen free radicals mechanisms in epidermis as a result of increased oxygen free radicals production. Glutathione S-transferase and thiobarbituric acid reactive substances were higher in all types of tumor than in healthy epidermis but oxidized glutathione was lower. Reduced glutathione and superoxide dismutase activity were lower in basal cell carcinoma and squamous cell carcinoma samples. Glutathione S-transferase increased, whereas superoxide dismutase and thiobarbituric acid reactive substances decreased in melanoma samples in direct relation to the Clark levels. Higher glutathione S-transferase activity, particularly in the most invasive forms of melanoma, indicates that this type of cancer is more malignant. Similarly, a decrease in superoxide dismutase activity can also encourage progression of the tumor. These results are in accord with those from tumor cell cultures and could suggest new strategies (gene therapy) for managing skin cancer. Key words: GSH/GSSG, GST, SOD, TBARS. *J Invest Dermatol 119:645–652, 2002

Reactive oxygen species (ROS) and oxygen free radicals (OFR) can modify several intracellular processes that, as a result, induce mutagenesis or impairment of biologic systems that, in turn, can affect cell survival. The most common OFR are the singlet oxygen, the hydroxyl radical, and the superoxide anion, all of which are normally present in skin because of physiologic metabolic oxidative processes which can increase in some circumstances, such as ultraviolet (UV) radiation (Meffert *et al*, 1976). Currently, there is a consensus that ROS and OFR are directly involved in numerous pathologic processes, including the initiation and promotion of events that lead to the development of cancer (Axelrod *et al*, 1990; Kehrer, 1993; Brar *et al* 2001).

Fortunately, living organisms have various defense mechanisms against ROS. These include OFR quenching, scavenging of damaged molecules, and repairing of molecular injuries, such as those that occur in DNA strands. Cells have nonenzymatic and enzymatic anti-oxidant protective mechanisms. Among the former are reduced glutathione (GSH), ascorbic acid, tocopherols, β-carotene, ubiquinone, and uric acid. These molecules can quench the OFR directly and, thus, prevent cellular damage. The enzymatic systems include catalase, superoxide dismutase (SOD), thioredoxin reductase, and the GSH-linked enzymes [GSH reductase, GSH peroxidase, and glutathione S-transferase (GST)]. GSH, therefore, has an important dual function in the protection against ROS: either as a direct scavenger of OFR or as a cofactor in the GSH-dependent enzyme systems. SOD is in the first line of defense. It converts the superoxide anion into hydrogen peroxide, which, in turn, can generate the extremely powerful hydroxyl radical as a result of the Fenton reaction in the presence of Fe^{2+}. Catalase, GSH, and GSH peroxidase prevent the hydroxyl radical from being generated by transforming the hydrogen peroxide into

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Abbreviations: BCC, basal cell carcinoma; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; MM, malignant melanoma; OFR, oxygen free radicals; SOD, superoxide dismutase; ROS, reactive oxygen species; SCC, squamous cell carcinoma; TBARS, thiobarbituric acid reactive substances.

H_2O and O_2 (Pré, 1991; Shindo *et al*, 1994). Lipoperoxidation by-products are an index of ROS production, especially that of hydroxyl radical production, and these can be reasonably reliably measured as thiobarbituric acid reactive substances (TBARS) (Janero, 1990; Pré, 1991).

Skin is a protective barrier against several external aggressive agents, especially UV radiation. It is therefore of considerable value to evaluate the activities of the above-mentioned defensive systems and to determine the extent of ROS production under different conditions. Several studies have reported the presence of GSH/GST and SOD systems in human skin (Raza *et al*, 1991; Kobayashi *et al*, 1993; Singhal *et al*, 1993; Giralt *et al*, 1996); however, most of these studies were conducted on whole skin or on a very limited number study samples and, further, the characteristics of these systems separately in epidermis and dermis have not been fully investigated. The epidermis and the dermis have different cell populations, vascularity, and intercellular constituents. Also, the epidermis is more exposed to external agents than is the dermis. Hence, it is to be expected that the extent of ROS production and the protective systems against ROS would be different. Shindo *et al* (1994), Applegate and Frenk (1995), and Kobayashi *et al* (1993) have reported on some of these differences but, to date, no large study exists of GST, SOD, GSH/oxidized glutathione (GSSG), and TBARS in human dermis compared with epidermis.

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common forms of skin cancer (Brand and Ackerman, 2000) and malignant melanoma (MM) is of utmost importance because of its malignancy. They have become more common in recent decades and the prevalence continues to increase (Austoker, 1994; Applegate and Frenk, 1995) probably as a result of our increasing exposure to UV rays. There is a considerable body of evidence that suggests that OFR-related systems contribute to the development of skin cancer and chemoresistance. Consequently, it has been suggested that some of these systems ought to be modified in order to improve response to cancer chemotherapy (see Discussion). As most of these studies have been conducted on animal and cell culture models, investigation involving spontaneous human skin tumors would provide new insight into the relationship between ROS and skin cancer on a molecular level and new therapeutic possibilities are opened up.

Hence, the main aim of this study was 2-fold: (i) to evaluate the activities of GST and SOD and the concentrations of GSH and TBARS in epidermis separate from dermis, and (ii) to measure these parameters in tissue samples of BCC, SCC, and MM, and to compare the results with those from healthy epidermis.

MATERIALS AND METHODS

Preparation and general treatment of samples Normal skin was obtained from different regions of the body (abdomen, thorax, back, face, neck, ear, extremities, and groin) from 51 men (mean age 54.7 ± 20.8 y) and 50 women (mean age 52.4 ± 19.6 y) who were undergoing surgery. The samples were saved in physiologic saline, delivered to the laboratory and processed on the same day. Their phototypes were mainly types III and IV, according to the Fitzpatrick classification and five cases were phototype II. Skin cancer samples included 21 BCC (13 nodular ulcerative, four superficial, two infiltrated and two sclerous), seven SCC (three moderately well differentiated and four well differentiated, of which two were with actinic keratosis), and 34 melanomas (18 superficial spreading melanoma, 10 lentigo maligna melanoma, three acral lentigenous melanoma, and three nodular melanoma). The samples were obtained from 36 male patients (69.7 ± 12.08 y) and 26 female patients (66.5 ± 16.9 y) and were taken mainly from the face, neck, ear, and nose. Their phototypes were types III and IV. Tumors were surgically excised as spindle-shaped pieces and sent, in sterile saline, to the pathology laboratory of each participating hospital where they were sectioned into parallel sagittal slices (about 2 mm thick) perpendicular to the longitudinal axis and submitted to the standard histopathology staining procedures for diagnosis. From those samples that were excess to requirements for diagnosis, portions of the fresh tumor material were removed from the central part of the slices, so as to avoid the surrounding nontumor material, and submitted to this study's analyses. All tumor samples were manipulated under cold saline and processed immediately, or stored at −80°C and processed within 1 wk.

The protocol was approved by the Ethics Committee of our University Hospital de Sant Joan de Reus (Universitat Rovira i Virgili) with anonymity of patient's data being guaranteed. Written informed consent was obtained from the patients prior to their tissue samples being included in this study's analyses.

In normal skin samples, the adipose tissue was carefully dissected out and the skin was cut into 0.5 × 0.5 cm pieces, which were immersed overnight in 0.25% trypsin in saline solution at 4°C. After a rinse in saline, the epidermis was separated from the dermis by gentle tugging with small tweezers. Five additional healthy skin samples were used to study the effect of trypsinization; each sample being divided in two parts with one being submitted to the trypsin procedure, whereas the other was frozen and the epidermis obtained by scraping with a scalpel. Tumor samples were finely minced with scissors. Except for the chromatofocusing analysis, whole skin, dermis, and tumor samples were homogenized in 200 mM, pH 6.25 sodium phosphate buffer (1 : 20 or 1 : 50; w/v) in a Polytron blender (Kinematica, Lucerne, Switzerland) cooled with crushed ice (speed 6, three strokes of 30 s each, at 30 s intervals). Epidermis was homogenized in a glass-Teflon Potter-Elvehjem homogenizer. Homogenates were centrifuged at 105,000 × *g* in an ultracentrifuge (Kontron, Milano, Italy) for 1 h to obtain the crude soluble supernatant on which the following measurements were performed.

Soluble proteins Soluble proteins were determined by the method of Lowry *et al* (1951) using bovine serum albumin as standard (Merck, Darmstadt, Germany).

GST activity V_{max} and K_m were calculated using spectrophotometric kinetic analysis with 1-chloro-2,4-dinitrobenzene as substrate at concentrations between 0.125 and 1.66 mM and GSH at a constant concentration of 2 mM (Habig *et al*, 1974). Spontaneous (background) activity (without enzyme) was subtracted for each substrate concentration.

GSH/GSSG GSH and GSSG were determined as described by Hissin and Hilf (1976) in a LS50 Perkin Elmer, Baconfield, UK fluorimeter at 350 nm and 420 nm (excitation and emission wavelengths, respectively).

SOD Total SOD activity was measured by the Misra and Fridovich (1972) method, which is based on the auto-oxidation of epinephrine at pH 10.2 and monitors the changes in absorption at 480 nm. One unit of SOD was defined as the equivalent amount of tissue or protein that caused a 50% inhibition of the epinephrine auto-oxidation rate.

TBARS TBARS were determined with a modification of the Buege and Aust (1978) method, in an LS50 Perkin Elmer fluorimeter at 515 nm and 548 nm (excitation and emission wavelengths, respectively) according to Richard *et al* (1992). Bis-diethyl-acetal malondialdehyde was used as a standard.

Chromatofocusing Samples were homogenized (1 : 10 or 1 : 20; w/v) as described above, but in a 20 mM Tris–HCl buffer (pH 7.2) containing sucrose (250 mM), GSH (1 mM), and Na_2 ethylenediamine tetraacetic acid (0.1 mM). Crude soluble extract was placed in a glass column (20 × 1 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden) filled with Polybuffer Exchanger 94 matrix, pre-equilibrated with imidazole (25 mM, pH 7.3) and eluted with Polybuffer 74. The whole procedure was carried out at 4°C. Fractions were recovered in an automated fraction collector and the GST activity was determined in a COBAS-MIRA centrifugal auto-analyzer (Roche, Basel, Switzerland) using 1-chloro-2,4-dinitrobenzene and GSH both at 1 mM concentration in the same buffer that was used for homogenization to correct for background/blank activity

Statistical analysis Statistical analyses were performed with the SPSS 10.0 program. The Kolmogorov–Smirnov goodness-of-fit test was used to determine the normality of the distributions. Differences between means were evaluated by the Student–Fisher t test for paired and nonpaired samples (normal distributions), based on the results of the variance analysis. For nonparametric (non-normal distributions) data, the Wilcoxon (paired samples) and the Mann–Whitney U test (nonpaired samples) were applied. Correlations between variables were analyzed by the Pearson correlation coefficient (r). In those melanoma samples in which there was sufficient tissue to conduct all the laboratory measurements, additional statistical analyses were performed to compare differences in the analytes with respect to Clark levels of tumor. These included ANOVA with the *post hoc* Scheffé test together with the

Table I. In healthy skin samples, there were numerous differences between dermis and epidermis for all parameters analyzed. In whole skin, intermediate values were found. Many differences were found between tumor samples and normal epidermis and between tumor samples and whole skin and the pattern depended on the type of tumor

Parameter	Whole skin	Dermis	Epidermis	SCC	BCC	MM
GST V_{max}						
nmol per min per mg protein	204.45 ± 133.6	60.6 ± 41.3	271.2 ± 166.6^{d}	341.23 ± 192.7^{e}	349.67 ± 310.4	372.6 ± 146.7^{c,h}
nmol per min per g tissue	1507 ± 1164	940 ± 684	6881 ± 4985^{d}*	9295 ± 6202^{g}*	9722 ± 14 433 ^{g}*	9671 ± 5558^{a,h}*
GST K_m (mM)	1.32 ± 0.5	1.26 ± 0.5	1.54 ± 0.7^{a}	1.48 ± 0.7	1.68 ± 0.9	1.51 ± 0.6
n	95	73	69	6	16	33
GSH						
μg per mg protein	10.24 ± 4.02	6.7 ± 2.2	12.0 ± 4.0^{d}	11.42 ± 9.26	9.3 ± 3.6^{a}	12.65 ± 4.0 ^{g}
μg per g tissue	71.31 ± 32.58	103.7 ± 37.1	300.6 ± 12.0^{d}	249.67 ± 156.9^{e}	181.5 ± 124.4^{c,g}	311.6 ± 146.8 ^{h}
GSSG						
μg per mg protein	12.85 ± 7.73	9.1 ± 3.3	20.1 ± 7.4^{d}	13.23 ± 7.61^{a}	15.17 ± 22.1	11.12 ± 6.2^{d}
μg per g tissue	89.29 ± 55.19	141.9 ± 63.3	505.5 ± 225.4^{d}	293.8 ± 128.3^{a,e}	177 ± 121.3^{d,e}	243.4 ± 104.6^{d,h}
GSH per GSSG	0.99 ± 0.57	0.81 ± 0.3	0.67 ± 0.3^{d}*	0.91 ± 0.5	1.16 ± 0.7^{a}*	1.76 ± 1.8^{d,f}*
n	95	73	69	6	15	33
SOD						
U per mg protein	29.64 ± 12.61	11.4 ± 4.9	16.7 ± 5.7^{c}	11.55 ± 8.32^{e}	10.33 ± 9.85^{a,g}	20.44 ± 7.7^{e}
U per g tissue	214.19 ± 86.68	176.4 ± 51.7	412.1 ± 109.2^{d}	249.3 ± 129.2^{a}	264.5 ± 213.5	424.9 ± 160.6 ^{h}
n	26	26	26	3	6	15
TBARS						
nmol per mg protein	1.13 ± 0.71	0.64 ± 0.3	0.14 ± 0.13^{d}	0.61 ± 0.18^{d}	0.451 ± 0.32^{a,e}	0.364 ± 0.17^{d,h}
nmol per g tissue	8.07 ± 3.74	10.1 ± 4.5	3.6 ± 2.9^{d}*	13.92 ± 2.6^{d,e}	10.09 ± 6.3^{a}	7.57 ± 3.6^{c}
n	26	26	22	3	8	15

Mean ± SD are represented separately for whole skin, dermis, epidermis, and tumor samples expressed either as per milligram of protein or as per gram of tissue. n: indicates the number of samples analyzed in each group of analytes. Results from dermis and epidermis were compared using nonpaired tests as the numbers of samples available for analyses were not always the same. When paired tests were used, the levels of statistical significance for paired samples (not shown) were of the same magnitude or higher than those presented here; p-values for the epidermis–dermis comparisons are indicated in the "epidermis" column with *a*, *b*, *c*, or *d* superscripts (< 0.05, < 0.01, < 0.005, or < 0.001, respectively). Tumor values were compared with those obtained in healthy epidermis using nonpaired tests (p-values are expressed as superscripts *a*, *b*, *c*, or *d* with values of < 0.05, < 0.01, < 0.005, or < 0.001, respectively). Tumor values were also compared with those obtained in whole skin using nonpaired tests and the p-values are expressed by *e*, *f*, *g*, or *h* superscripts, corresponding to < 0.05, < 0.01, < 0.005, or < 0.001, respectively. The absence of superscript means the differences were not statistically significant. (*)indicates a statistical significance obtained by the Mann–Whitney U test. All other comparisons are with unpaired Student t test.

multinomial logistic regression analysis. Results were considered statistically significant when $p < 0.05$.

RESULTS

After some preliminary validation studies, we were only able to study all the OFR-related parameters in those samples for which there was enough tissue remaining. It was not possible therefore to determine all of the parameters in all of the samples. These discrepancies in overall numbers of samples assessed are highlighted in the results tables, where applicable.

In healthy samples, significant differences were observed between dermis and epidermis. GST values (V_{max} and K_m), SOD activity, GSH, and GSSG were higher in the epidermis than in the dermis, whereas TBARS were lower.

Table I shows that the maximal difference between the two skin layers was observed for GST V_{max} (seven times higher per gram of tissue) and the minimal difference was observed for SOD activity (1.5 times higher per milligram of protein). GST affinity was only slightly lower, albeit statistically significant ($p = 0.016$), in the epidermis than in the dermis (higher K_m value). Conversely, TBARS were 4.6 times higher in the dermis than in the epidermis when expressed as per milligram of protein. The results observed in whole skin samples were in the range between those values obtained in the separate skin layers (see **Table I**).

In the epidermis, statistically significant positive correlations were observed between GST V_{max} and several parameters: the K_m value ($r = 0.36$; $p = 0.002$), SOD activity ($r = 0.68$; $p < 0.001$), GSH content ($r = 0.41$; $p < 0.001$), and GSSG content ($r = 0.38$; $p = 0.001$). SOD activity correlated positively with the GSH content ($r = 0.44$; $p = 0.027$), whereas the amount of GSH was negatively correlated with TBARS ($r = -0.48$; $p = 0.024$).

In the dermis, there were statistically significant positive correlations between GST V_{max} and K_m values ($r = 0.33$; $p = 0.004$) as well as between SOD activity and TBARS ($r = 0.39$; $p = 0.048$). In turn, TBARS correlated negatively with the GSSG content ($r = -0.47$; $p = 0.015$).

OFR-related parameters in skin cancer samples were significantly different from healthy epidermis and whole skin but depended on the type of tumor **Table I** also shows the results from tumor samples together with those from normal epidermis and whole skin. GST V_{max} was higher in all tumor samples than in normal epidermis and whole skin. In BCC and SCC this increase was only significant in comparison with whole skin samples, especially when expressed as per gram of tissue. GSH content was roughly similar in all three types of tumors and only significantly lower in BCC samples than in healthy epidermis when expressed as per milligram of protein. All tumors showed higher GSH values than normal whole skin, when expressed as per gram of tissue. GSSG content was lower in all tumors than in healthy epidermis but clearly higher than in whole skin when expressed as per gram of tissue. These differences influenced the value of the GSH/GSSG ratio, which was significantly higher in BCC than in normal epidermis and also higher in MM than in normal epidermis and whole skin. In BCC and SCC samples, SOD activity was lower than in epidermis, but particularly low in comparison with whole skin when expressed as per milligram of protein. In MM samples, SOD was not different from epidermis but higher than in whole skin when expressed as per gram of tissue.

Finally, TBARS were clearly higher in all tumor samples, especially in the SCC group, than in healthy epidermis. This was not the case in comparison with whole skin, especially when expressed as per milligram of protein.

In melanomas, GST V_{max}, SOD activity, and TBARS content depended on the Clark level of the tumor In the 15 samples in which there was sufficient tissue to measure all the proposed analytes, GST activity increased progressively as the Clark level increased and the mean values ranged between 261 (level II)

Table II. In melanoma samples, OFR-related parameters depended on the Clark level

Clark level	II (n = 6)	III (n = 6)	IV + V (n = 3)	p
GST V_{max}				
nmol per min per mg protein	261.8 ± 123.4	377.4 ± 147.5	409.0 ± 96.3	NS
nmol per min per g tissue	5774 ± 3083	8670 ± 5742	9620 ± 3636	NS
SOD				
U per mg protein	24.8 ± 5.6	21.7 ± 7.4	10.3 ± 0.5	0.016
U per g tissue	517.2 ± 181.1	423.9 ± 83.0	238.1 ± 31.5	0.032
TBARS				
nmol per mg protein	0.53 ± 0.2	0.33 ± 0.07	0.18 ± 0.06	0.007
nmol per g tissue	10.6 ± 4.2	7.0 ± 2.7	4.2 ± 1.4	0.04
GST/SOD	11.7 ± 7.8	20.1 ± 13.9	39.7 ± 10.2	0.013
GST/TBARS	593.7 ± 432.4	1124 ± 432.4	2352 ± 804.5	0.001

Results (mean ± SD) are grouped with respect to the Clark level of melanomas (n = 15). The values are expressed as per milligram of protein or as per gram of tissue. p-values were obtained from the ANOVA test. *Post hoc* analysis by the Scheffé test indicated that the significance lay only between levels II and IV + V for the values of SOD, TBARS and GST/SOD, whereas the differences in the GST/TBARS ratios between all the three levels were statistically significant.

Table III. The multinomial logistic regression analysis demonstrated that OFR-related parameters were good predictors of the Clark level in melanoma samples

Parameters	p	% correct	Incorrect cases
GST + SOD + TBARS per mg of protein	0.001	93.3	1 level II case, expected for level III
GST + SOD + TBARS per g of tissue	< 0.001	100	0 cases
GST/SOD + GST/TBARS	0.001	86.7	1 level II case, expected for level III 1 level III case, expected for level II

The statistical significance (p) was calculated from the –2 log-likelihood ratio test in the best-fit final model for each of the three groups of variables: GST + SOD + TBARS both as per milligram of protein as well as per gram of tissue, and GST/SOD + GST/TBARS. The Clark level and number of samples are as in **Table II**.

and 474 nmol per min per mg protein (level V); SOD activity decreased from a mean value of 25 (level II) to 10 U per mg protein (level V); and TBARS decreased from a mean value of 0.53 (level II) to 0.14 (level V) nmol per mg protein.

To evaluate these differences further we performed an ANOVA analysis on the results from these samples (n = 15). We grouped samples with Clark levels IV (n = 2) and V (n = 1) together to meet the minimum statistical requirements (n = 3). (It is difficult to obtain samples with high Clark levels as they are, fortunately, quite rare.) As can be observed in **Table II**, TBARS content and SOD activity significantly decreased as the Clark level increased. The GST activity increased, although it did not reach statistical significance. The inverse relationship observed between GST activity and SOD activity and between GST activity and TBARS content in the three Clark levels made it possible for us to create two new variables, i.e., GST/SOD and GST/TBARS ratios. The values obtained with these new variables also showed a clear relationship with the Clark level, the ratios being lowest at level II and highest at level IV + V. The ANOVA analysis of these variables demonstrated significant differences between groups, particularly in the case of the GST/TBARS ratio (see **Table II**).

Soluble protein content in the three Clark groups was not significantly different. Finally, multinomial logistic regression analysis was carried out using the Clark level as the dependent variable (levels II, III, and IV + V) and the GST activity, SOD activity, TBARS content, GST/SOD, and GST/TBARS as predictor variables. The −2 log-likelihood test for these variables demonstrated that they were all good predictors of the Clark level, as can be seen in **Table III**.

Chromatofocusing analysis showed that the two major GST isoenzymes were the basic and the acidic forms The acidic GST form was eluted from the column as a clearly identified peak. In healthy dermis and epidermis, this form made up slightly more than 50% of total activity. The basic isoenzymes that eluted off the column had a more heterogeneous pattern and made up about 45% of total activity in both the dermis and the epidermis. Neutral GST isoforms, which also appeared in some samples, made up only 3–4% of total activity. In all tumor samples (five BCC, one SCC, and one MM) the acidic activity increased to about 80%, whereas basic forms dropped to 20% of the total GST activity. In four BCC samples, the neutral activity was found to be 2%. **Table IV** summarizes these results and **Fig 1** shows the chromatofocusing profile of the epidermis and the BCC samples.

DISCUSSION

In healthy samples, the results clearly show that the three protective systems studied (GST, GSH/GSSG, and SOD) were higher in epidermis than in dermis. This could be the result of the different cell patterns of the two skin layers or of an adaptive response, as the epidermis is more exposed to environmental agents than the dermis. This implies that the metabolic processes and the production of endogenous OFR of the two skin layers are different.

The main GST isoenzymes and the relative activities of the various peaks obtained by chromatofocusing were similar in the dermis and the epidermis (**Table IV**) and the characteristics of the dermal GST system (i.e., V_{max} and K_m) are similar to those we described in the human sebaceous gland (Giralt *et al*, 1996). Hence, the K_m value of GST may be higher in epidermis than dermis not because of isoenzyme differences in the two skin layers but because of a greater production of some OFR in epidermis, which would tend to lower the affinity of the enzyme. On the other hand, the greater SOD activity observed in the epidermis may be a response

Table IV. Acidic and basic GST isoforms were predominant in healthy samples. The acidic form was significantly increased in all tumors

Sample	Basic	Neutral	Acidic
Dermis n = 3	47.1 ± 13.4 (3)	3.9 (1)	51.4 ± 14.4 (3)
Epidermis n = 5	42.6 ± 16.6 (5)	3.1 ± 2.1 (2)	54.2 ± 36.1 (5)
Tumors n = 7	19.2 ± 8.2 (7)	2.2 ± 1.0 (4)	77.2 ± 8.2 (7)

Mean ± SD of the percentage of GST activity of the different isoenzymes eluted from the chromatofocusing column. The numbers of samples that show activity for each isoform are in parenthesis.

to a higher production of superoxide anion. So, if this radical is neutralized by the SOD system, more H_2O_2 will be produced, which in turn may determine the formation of hydroperoxides. Consequently, there ought to have been more TBARS in epidermis than in dermis, but this was not so. This apparent discrepancy may be explained by the greater activity of the protective systems found in epidermis determining a more favorable balance between anti-OFR systems and TBARS content than in dermis. In a pilot study, Shindo *et al* (1994) reported differences in SOD activity in the dermis and epidermis that were identical to those observed in our study; albeit their absolute values were different from ours, probably due to methodologic differences. Additionally, they found that other enzymes (GSH reductase, GSH peroxidase, and catalase) were higher in the epidermis than in the dermis. In their study, catalase activity, in particular, was between four and eight times higher when expressed as per milligram of protein and as per gram of tissue, respectively. Overall, this explains, in part, why TBARS are lower in epidermis than in dermis. The evaluation of the lipoperoxidation by-products using the original colorimetric TBARS analysis may be open to criticism due to lack of specificity of the method, especially when the purpose is to measure malondialdehyde formation. The use of fluorimetry to measure TBARS (Richard *et al*, 1992) increases specificity and sensitivity, although by-products other than malondialdehyde are also TBARS. The purpose of our study was to evaluate not only malondialdehyde but also many other by-products resulting from the reaction with hydroperoxides; thus, the low specificity of the TBARS reaction can be considered, under the present circumstances, as an advantage, especially as it is a simple and rapid method. In summary, it seems reasonable to accept that the more developed anti-OFR systems in normal epidermis are a physiologic consequence of a greater production of OFR than in dermis. When specific defense mechanisms work efficiently, damage to specific molecules is prevented (lower TBARS content in epidermis than in dermis), but any imbalance in the production–inhibition of OFR will increase the risk of degenerative processes such as epidermal cancers.

Before discussing the tumor results in detail, two aspects need to be highlighted. First, it seems more appropriate that they should be compared with normal epidermal samples because of their epidermal origin and because both types of samples presented similar amounts of protein per gram of tissue. So, we shall base the following section of the discussion above all on the healthy epidermal samples (though comparisons between tumor samples and whole skin can be drawn from **Table I**). Secondly, tumor samples were not treated with trypsin as had the normal samples. Hence we needed to verify that this treatment did not alter the analytical measurements in healthy skin. The values obtained from frozen whole skin and dermal samples (after the epidermis had been scraped off) were similar to those obtained after trypsin incubation (results not shown). On the other hand, in epidermal samples obtained by scraping, the amount of soluble protein recovered was considerably lower than when samples were obtained by trypsinization. So we concluded that the freezing and scraping procedure destroyed a significant number of epidermal cells and therefore led to a loss of soluble fraction. As such, the trypsinization procedure is more appropriate than the freezing–scraping technique for separating dermis from epidermis and for performing comparisons between the skin layers as well as between tumor samples and healthy epidermis.

In all tumor samples, GST activity was higher than in healthy epidermis but the difference was only statistically significant in MM samples, probably due to the small number of BCC and SCC samples. Chromatofocusing results demonstrated that the greater GST activity in tumor samples was mainly due to the increase in the pi isoform. TBARS were 2.6–4.3 times higher than in healthy epidermis. GSH content and SOD activity were lower than in normal epidermis only in BCC and SCC samples. The ANOVA analysis (**Table II**) and the multinomial logistic regression analysis (**Table III**) demonstrated some interesting relationships between OFR-related parameters and the stage of MM samples: GST activity increased with the Clark level, whereas the amount of TBARS and SOD activity was inversely related to the Clark level. Moreover, the GST/SOD and GST/TBARS ratios also increased with the Clark level. Finally, the multinomial logistic regression analysis also confirmed that all three parameters and their corresponding ratios are very good predictors of the Clark level. To summarize, tumor samples were characterized by an increase in GST activity and TBARS content, and by a decrease in SOD activity.

Previous reports related to these parameters should now be discussed. GST has been shown to increase in many human tumor tissues or tumor cell lines (Mannervik *et al*, 1987; Shea *et al*, 1988; Lafuente *et al*, 1990; Zhang *et al*, 1994), and in normal skin after UV irradiation (Iizawa *et al*, 1994), especially in the pi isoform. In this respect, the GSH/GST system is considered part of the aptly termed multidrug resistance system, such as P-glycoprotein and topoisomerase II. So, tumor cells with high GST activity are considered to be particularly resistant to anti-neoplastic drugs, as is the case in melanoma-derived cell lines (Serrone and Hersey, 1999). Also, the overexpression of GST in tumor cells seems to be linked to the expression of such oncogenes as the mutated N-Ras oncogene, which has recently been implicated in melanoma resistance to cisplatin; the roles of two other oncogenes, Bcl-2 and p53, are currently being investigated (Serrone and Hersey, 1999). The GST pi gene has been mapped in the human chromosome to 11q13 (Moscow *et al*, 1988), a region in which the putative proto-oncogenes int2 and hst1 have also been mapped (Burgess, 1988; Brookes *et al*, 1989). Consequently, the increase in GST activity has been considered not only as a tumor marker (Tsuchida and Sato, 1992) but also as an index of malignancy, which includes a greater aggressiveness of the tumor and a poorer prognosis (Gilbert *et al*, 1993). van Iersel *et al*, (1999) described the inhibitory effects of prostaglandin A2 on cell proliferation in cultures of IGR-39 melanoma cells and concluded that these effects are probably mediated by an inhibitory effect on the GSTP1-1 isoenzyme.

In contrast to these negative effects of GST, the lack of GST expression (GSTM and GSTT null genotypes) together with low enzyme activities could contribute to the development of several tumors in humans (Heagerty *et al*, 1996; Lear *et al*, 1996, 1997; Yengi *et al*, 1996; Clapper and Szarka, 1998). As such, GST may be considered as a Janus-type system, i.e., they are believed to be protective when specific genotypes are present but, conversely, are predictors of poor prognosis when the enzyme is overexpressed (as is the case in our MM samples) because of the resistance to chemotherapeutic regimens.

SOD activity appears to be inversely related to the proliferation of malignant cells. In cultures of IPC182 and IGR221 melanoma cells, the end of the exponential growth phase coincided with the maximal expression of SOD together with a decrease of catalase and GSH peroxidase activities (Bravard *et al*, 1999). These authors also suggest that alterations in anti-oxidant enzyme expression, especially the decrease in the SOD system, are involved in the

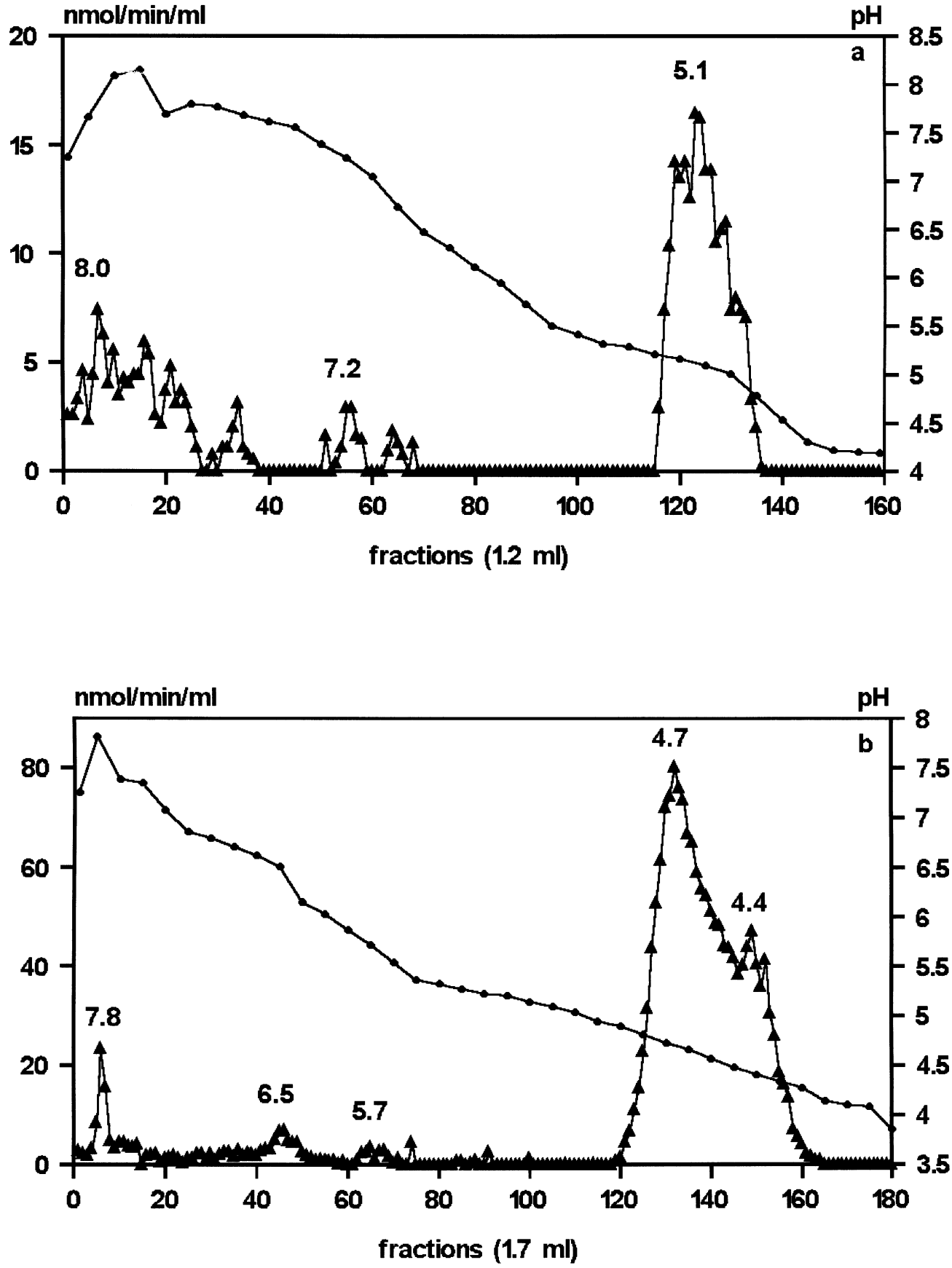

Figure 1. Chromatofocusing analysis of GST activity revealed an increase in the acidic isoform in tumor samples. GST activity was calculated in each eluant fraction (1.2 or 1.7 ml) from the column. The pH was determined every five fractions. The pH values of the peaks are noted on the chromatofocusing profile. (*a*) Normal epidermis; (*b*) BCC sample. Triangles: GST activity (mU per ml); circles: pH values.

proliferation and differentiation of melanoma cells. Picardo *et al* (1996) reported that melanoma cells showed an inverse relationship between SOD and catalase activities, whereas in another study (Grammatico *et al*, 1998) it was concluded that alterations in the scavenger systems were involved in the development of MM. Bittinger *et al* (1998) demonstrated that the production of superoxide anions by melanoma cells was involved in the mechanism of metastasis. In human melanoma cells, the increase in SOD downregulates ICAM-1 expression, which is important in the expansion process of the tumor (Morandini *et al*, 1999). The tumor promoter tetradecanoyl-phorbol-acetate inhibits SOD activity in mouse skin and silimaryn prevents tumor development and SOD depletion (Zhao *et al*, 1999). The topical application of SOD protects against psoralen + UVA-induced inflammatory reactions in murine and human skin (Filipe *et al*, 1997).

Finally, it has been demonstrated that there is a relationship between UV exposure, skin cancer, and a TBARS increase (Meffert *et al*, 1976; Setlow *et al*, 1993). Increase of polyunsaturated fatty acids and an imbalance in the OFR/protective systems in MM cells leads to a higher production of lipoperoxides (Picardo *et al*, 1996; Grammatico *et al*, 1998).

The inverse relationship observed between GST and SOD activities found in our tumor samples is concordant with previous reports. BCC and SCC presented less SOD activity than normal epidermis. The observation that the increase in GST activity in these tumors was not significantly different from that in healthy epidermis may be due to the small number of samples studied but it can also reflect a lesser malignancy in comparison with melanoma. In MM samples, a higher GST activity was found in all cases and an inverse relationship between GST and SOD activities was observed as the Clark level increased. This is in agreement with the higher malignancy of this type of tumor, especially those of the highest Clark levels.

TBARS were lower in the more invasive forms of MM. This could be because SOD activity decreases (and less hydroperoxides are formed) or because there is an increase in catalase and GSH peroxidase at these stages (see Bravard *et al*, 1999).

The observation that the parameters studied changed with the Clark level cannot be attributed to the presence of contaminating nontumor tissue (e.g., more dermis in the higher Clark level tissue) as the tumor samples were carefully selected to be free of surrounding nontumor tissue. Moreover, the soluble protein content was identical in all melanoma samples, independently of the Clark level.

It has been suggested that inactivating the GST system would be a way of increasing the sensitivity of neoplastic cells to chemotherapy (Lafuente *et al*, 1990; O'Dwyer *et al*, 1991; Khadir *et al*, 1999). There have been no clinical studies to support this approach however. It is hoped that gene therapy related to other anti-OFR systems will be a viable strategy in the treatment of MM (Bravard *et al*, 1999). For example, Church *et al* (1993) showed that transfecting MnSOD cDNA into human melanoma cells increased enzyme expression and suppressed their malignant phenotype. Rieber and Rieber (1999) reported the retroviral transfer of the anti-sense cyclin D1 gene to differentiation-refractory K1735 melanoma, which, among other changes, leads to the loss of tumorigenicity *in vivo* and a greater SOD/peroxidase ratio; both being associated with replication senescence. Also, transfection of a human MnSOD cDNA into the promotable mouse epidermal cell line JB6 clone41 elicits an overexpression of the enzyme and induces a slower growth rate of the cells (Amstad *et al*, 1997). Blocking oxidant activation of transcription factors such as the nuclear factor-κB (Brar *et al* 2001), also represents an interesting strategy.

In conclusion, the present results obtained from clinical samples coincide with those obtained in cancer cell cultures and animal models. This is of considerable importance in the better understanding of the molecular factors involved in tumor development, especially those related to OFR and, as such, new strategies, including gene therapy, may be envisaged for the treatment of skin cancer.

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