Oxygen Levels in Normal and Previously Irradiated Human Skin as Assessed by EF5 Binding

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The oxygen status of skin is a controversial topic. Skin is radiosensitive, suggesting it is well-oxygenated. However, it can be further sensitized with nitroimidazole drugs, implying that it is partially hypoxic. Skin oxygen levels are difficult to measure with either electrodes or the hypoxia-monitoring agent ^{3}H-misonidazole. For the latter, binding has previously been reported to be high in murine skin, but this could be attributed to either non-oxygen-dependent variations in nitroreductase activity, drug metabolism, and/or actual oxygen gradients. We obtained tumor and skin from patients given EF5, a 2-nitroimidazole tissue hypoxia monitor. We performed immunohistochemical studies using highly specific monoclonal antibodies for the hypoxia-dependent production of EF5 tissue adducts. Some tissue sections were counterstained using either Ki67 for proliferation or CD31 for vessels. We found that the human dermis is well-oxygenated, the epidermis is modestly hypoxic and portions of some sebaceous glands and hair follicles are moderately to severely hypoxic. Normal and irradiated skin had similar oxygenation patterns. Control studies demonstrated that these observations are not due to tissue variations in nitroreductase activity. The importance of the highly heterogeneous distribution of oxygen in skin requires further study, but recent investigations suggest that skin hypoxia may have important clinical ramifications including mediating cellular transformation.

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INTRODUCTION

The oxygen level in human skin is of considerable interest to scientists and physicians in a diverse range of fields. Anesthesiologists are interested in skin oxygenation as a monitor of systemic oxygenation. Wound healing or its absence is known to be affected by tissue oxygenation (Hunt *et al.*, 2004) and this is of concern to surgeons. Radiation oncologists have long known that tissue oxygenation affects the extent of radiation damage; the skin overlying irradiated volumes is at substantial risk for complications during radiation treatment of superficial tumors, for example breast cancer. Acute radiation side effects include erythema and desquamation; hair loss occurs during or shortly after therapy. Late radiation effects include fibrosis, telangiectasia, and secondary malignancies. The etiology of the late radiation effects is not well defined, especially related to oxygen dependence. However, there are data to suggest that long-term production of reactive oxygen species within the irradiated site may be important (Moeller *et al.*, 2004). The efficacy of other cancer treatment modalities such as chemotherapy and photodynamic therapy is also known to be modified by tissue oxygenation (Yamagata *et al.*, 1992; Busch *et al.*, 2002).

There have been numerous oxygen partial pressure (pO_2) studies of skin in animal models, particularly rodents and pigs. However, the direct measurement of pO_2 in unperturbed human skin has been limited by the lack of appropriate methodology. Electrode and other studies have emphasized the pO_2 in wounded skin and confirmed the presence of hypoxia in this situation (Koch *et al.*, 1997; Hunt *et al.*, 2004). Needle electrode measurements in normal skin are difficult because of the skin's highly heterogeneous composition, its thinness in mice, and its toughness in all mammals. Indirect information on the pO_2 of human skin comes from its response during radiation therapy. The skin readily manifests acute radiation damage, inferring that the proliferating components of the skin are well-oxygenated. This is also true for hair production and growth, as hair regrowth is limited following radiation treatment. Extensive studies by Cobb *et al.* have documented the uptake of the prototypic metabolic hypoxia detector ^{3}H-misonidazole in epidermis and various appendages of rodent skin (Cobb and Nolan, 1989; Cobb *et al.*, 1989, 1990b). However, the conclusion reached in these studies was that this and similar binding in other ''aerobic'' rodent tissues was artifactual due to high nitroreductase activity (Cobb *et al.*, 1990c). In contrast, *in vivo* studies from other laboratories showed that high misonidazole binding in esophagus could be inhibited

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Abbreviations: AUC, area under the curve; CRB, cube reference binding; pO_2, oxygen partial pressure

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by 95% oxygen breathing (Parliament *et al.*, 1992). More recently, pO_2 values of <5 mm Hg have been recorded in mouse dermis using electron paramagnetic resonance techniques (Krzic *et al.*, 2001). Thus, the question of the actual pO_2 of skin remains unresolved for both animals and humans.

EF5 is a 2-nitroimidazole drug that was specifically designed to monitor tissue hypoxia. It has been validated for quantitative studies of both neoplastic and non-neoplastic tissue pO_2 in humans and in animal models (Koch *et al.*, 1995, 2001; Evans *et al.*, 1996, 2004b, 2006; Bergeron *et al.*, 1999; Koch, 2001). Herein we present data based on EF5 binding as a measure of tissue oxygenation in irradiated and untreated human skin. Our findings support the extensive literature on the radiation sensitivity of the skin because we find that the majority of the skin, that is, the dermis is oxic. However, EF5 binding is present to varying degrees in human epidermis and other skin appendages, including portions of some hair follicles and sebaceous glands. In order to determine whether this heterogeneity was oxygen dependent *versus* an artifact of nitroreductase activity or nonspecific binding, we performed control studies to assess the maximum tissue binding under conditions of severe oxygen deprivation. These data were obtained by incubating small tissue cubes under 0.02% oxygen with EF3, a sister compound of EF5. This procedure is called the ''cube reference binding'' (CRB) study (Koch, 2001; Evans *et al.*, 2004b). We hypothesize that all skin appendages in a given patient will bind EF3 equally under the CRB condition and, therefore, any variations of binding *in vivo* would reflect true oxygen differences. The resultant data are consistent with the conclusion that gradients of EF5 binding result from gradients in oxygen tension rather than variations in nitroreductase activity. However, the latter contributes substantially to patient-to-patient variability in absolute binding and, therefore, must be taken into account in order to obtain a reasonable estimate of actual tissue pO_2.

RESULTS

Skin and tumor tissues from 19 patients were assessed. Seventeen patients were receiving surgery for head and neck cancer and two were being treated for extremity sarcoma (Table 1). Ten patients had received radiation therapy preceding the surgery at which the tumor and skin tissue were removed. Skin from two patients who did not receive EF5 was available for control studies of CRB (see below for methodological and analytic methods).

Table 1. Patient information

PT ID no.	Sex	Age (years)	Race	Previous radiation therapy	Months[1]	Tumor site	Skin type studied
66	M	47	C	Yes	3	H&N	Normal and XRT
67	M	62	C	No	0	H&N	Normal
73	M	65	C	Yes	7.5	H&N	XRT only
75	M	48	C	Yes	24	H&N	XRT only
79	F	60	C	Yes	10	H&N	Normal and XRT
80	M	45	C	No	0	H&N	Normal
81	M	71	C	No	0	H&N	Normal
83	M	70	C	No	0	H&N	Normal
85	M	57	C	No	0	H&N	Normal
91	M	63	C	Yes	3	Sarc	XRT only
92	M	66	A	Yes	1	Sarc	XRT only
93	M	63	C	Yes	4	H&N	Normal and XRT
94	M	60	C	Yes	8.5	H&N	Normal and XRT
95	F	59	C	Yes	9	H&N	XRT only
97	M	74	C	No	0	H&N	Normal
98	M	64	C	Yes	18	H&N	XRT only
102	M	69	C	No	0	H&N	Normal
103	F	62	C	No	0	H&N	Normal
104	M	68	C	No	0	H&N	Normal
105[2]	M	65	C	—	—	—	—
106[2]	F	54	C	—	—	—	—

A, Asian; C, Caucasian; H&N, head and neck cancers; Sarc, sarcoma; XRT, radiation therapy.
[1]From end of XRT to biopsy.
[2]CRB studies only.

In situ EF5 binding

Skin is a very complex tissue (Figure 1a). Cell density and type vary dramatically between the epidermis, dermis, and the various skin appendages. The typical pattern of *in situ* EF5 binding seen for normal skin (Figures 1 and 2) includes a layer of epithelial cells within the superficial epidermis with moderate EF5 binding. The binding gradient was substantial with the highest binding below the skin surface in the stratum granulosum. Only viable cells containing a nucleus bound EF5, so binding was absent from the metabolically inactive cells in the stratum corneum and lucidum. Variable binding was also seen in portions of some sebaceous glands and in an annular region surrounding the shafts of some portions of some hair follicles. It is not possible to depict the large range of EF5 binding in a simple black and white image. Therefore, the rectangular insets in Figure 2a illustrate a five-fold electronically enhanced signal in the epidermis and a relatively low-binding hair follicle. Very little EF5 binding was seen in the dermis and portions of many skin appendages. Similar *in situ* binding patterns were observed in irradiated skin (Figure 2c and d).

A summary of the absolute fluorescence intensities for the *in situ* binding of skin samples from all patients indicated very substantial inter- and intra-patient heterogeneity (Table 2a). However, consistent patterns did emerge (Figures 1 and 2). For example, binding in the dermis was always much lower than that of the other skin appendages (epidermis, glands, hair follicles). Substantial variation occurred in these three substructures, even within an individual patient's tissue. Thus, not all sebaceous glands and hair follicles contained high binding. Even in samples where there was very bright binding around some hair shafts, large gradients of binding away from the hair shaft or sebaceous glands were observed.

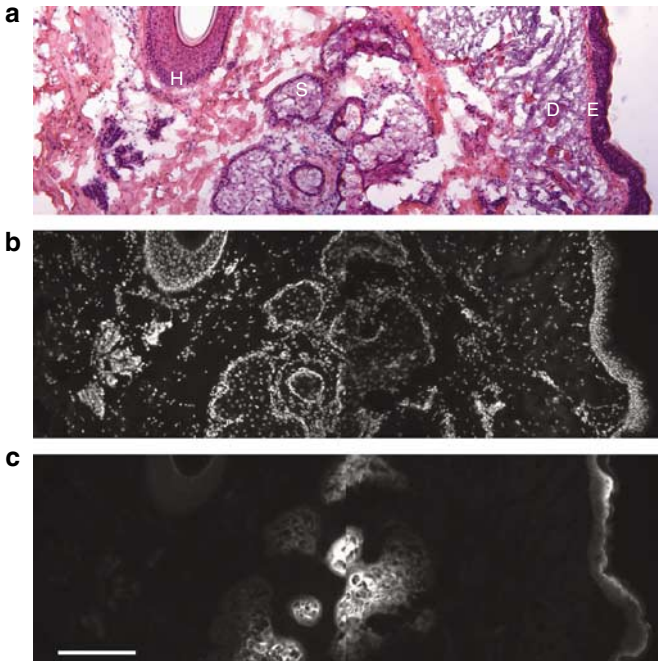

Figure 1. The complexity of skin and variations in cell density are demonstrated in these images of normal human skin. (**a**) Stained with hematoxylin and eosin, (**b**) counterstained with Hoechst 33342 to demonstrate the location of nuclei, and (**c**) the EF5 binding patterns (bar = 0.25 mm). Examples of hair follicle (H), sebaceous gland (S), dermis (D), and epidermis (E) are identified in the hematoxalin and eosin image.

Control studies including CRB

Various control studies were performed in order to determine whether the variations in EF5 binding (Figures 1 and 2, Table 2a) reflected variations in tissue pO_2 *versus* non-oxygen dependent binding artifacts. The etiology of such

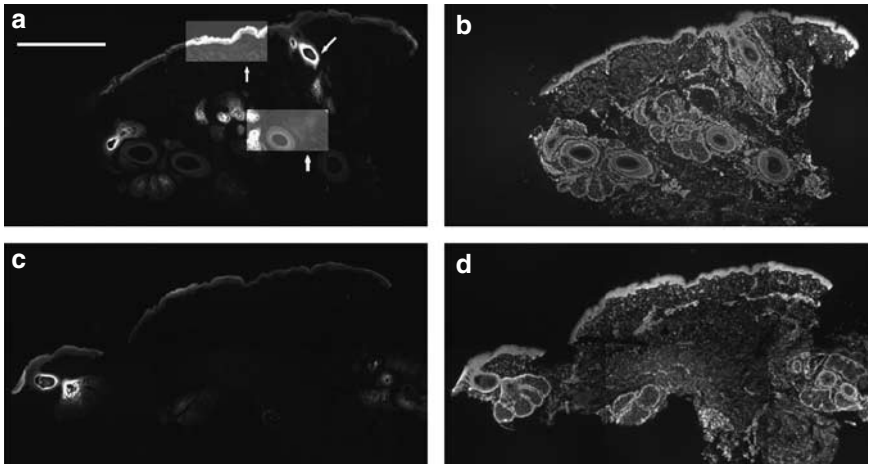

Figure 2. EF5 binding in irradiated and normal skin. (**a**, **b**) EF5 binding in nonirradiated and (**c**, **d**) irradiated skin. Bar = 1.0 mm. Panels a and c show the patterns of EF5 binding and Panels b and d show the patterns of nuclei, based on Hoescht 33342 counterstaining. In both normal and irradiated skin, EF5 binding in dermis $\ll$ epidermis $<$ sebaceous glands $\approx$ hair follicles. However, high binding is only found in some hair follicles and sebaceous glands, or in portions thereof. The fluorescence intensities in the rectangular insets of (**a**) have been electronically increased by a factor of 5 to show details of the tissue (vertical arrows). In the upper electronically enhanced rectangle, the contrast between epidermis and dermis is quite striking. Note that the off-tissue intensity is still lower than the lowest values of the dermis. Despite the five-fold fluorescence enhancement, the cells surrounding the hair shaft depicted in the lower rectangle are at much lower binding levels than those surrounding the hair shaft in the upper right image (not adjusted, diagonal arrow).

Table 2a. Summary of *in situ* EF5 binding using absolute fluorescence

Patient ID no.	Site	Absolute CRB value	Drug AUC	Average *in situ* skin binding
67	H&N	612	1.47	408
79	H&N	1,916	1.20	13–92
85	H&N	687	1.45	73–78
91	Lower extremity	361	1.54	73
92	Lower extremity	308	1.63	68
93 (control)	H&N	1,706	1.63	284
93 (irradiated)	H&N	2,152	1.63	98
94	H&N	950	1.44	137–333
98	H&N	583	1.36	215–237
102	H&N	1,048	2.04	454
103	H&N	709	1.38	44

AUC, area under the curve; CRB, cube reference binding; H&N, head and neck.
Absolute CRB values are the average skin binding, corrected only for microscope exposure time and camera optics. To translate these absolute values into tissue oxygen concentration, they must be corrected for drug AUC (in mM hours), and cube reference binding (CRB; see Tables 2c and d).

variations may include (1) tissue autofluorescence, (2) nonspecific antibody binding, (3) tissue variations in nitroreductase activity, or (4) non-oxygen dependent metabolism of drug. Data providing insight into these potential sources of variation are discussed below:

Autofluorescence. We have found that the methods we use do not produce tissue autofluorescence at wavelengths appropriate for Cy3 (excitation = 541–551 nm and emission = 590LP nm; Nikon G-1B filter set). We tested for this on all samples by imaging an unstained tissue section (data not shown). One caveat is that surgical inks or tissues containing tattoos can produce ultrabright spots of fluorescence that interfere with our quantitation techniques (see Materials and Methods). Autofluorescence did increase at lower excitation wavelengths, for example, those used for Hoechst 33342 or FITC. This was not problematic for use of Hoechst 33342 (UV excitation) to derive nuclear-DNA-based tissue masks because the Hoechst-DNA emission was uniformly bright.

Nonspecific antibody binding. Use of the anti-EF5 antibody (ELK-51) produced only background levels of fluorescence in tissues from patients who were not treated with EF5 (patients no. 105 and 106; data not shown). It is impractical to obtain biopsies from patients prior to EF5 administration, so we devised an analytic method to provide the equivalent of nonspecific antibody staining. This process is detailed in the Materials and Methods section below. Briefly, a representative section from each patient was stained with the ELK 3-51 mixed with an excess of authentic EF5 drug (0.5 mM). The excess drug binds to all specific sites on the antibody and allows for only nonspecific binding. The fluorescence values obtained from this control (termed ''Competed Stain'') were about 100-fold less than the brightest binding areas of the slides stained under standard conditions with noncompeted ELK 3-51 antibody (''Regular Stain''). For data analysis, the Competed Stain values were subtracted from the absolute fluorescence values for the Regular Stain sections.

EF5 adducts formed under hypoxic conditions are covalent and therefore stable over short periods of time. However, they are eventually lost due to protein turnover. One patient reported herein had skin studies performed twice. This patient received EF5 preceding the first surgery and skin was removed for analysis of EF5 binding. Additional surgery in the head and neck region was required 5 months later but EF5 was not administered at that time. As with patients who had never received EF5 (patients no. 105, 106), anti-EF5 binding not was observed in the second biopsy of this patient.

Tissue variations in nitroreductase values. The dual purposes of the CRB studies were to first determine whether the various skin substructures (dermis, epidermis, hair follicles, sebaceous glands) had differing abilities to reduce and bind EF5 (due to differing nitroreductase levels) and second, to determine the maximum binding levels possible for each patient's tissues. These CRB studies were performed with EF3. EF3 and EF5 differ only in the number of sidechain fluorines (3 or 5, respectively) and have essentially identical binding characteristics when measured *in vitro* (Koch, 2001). We use EF3 for the CRB studies because it is possible to distinguish the EF5 adducts produced during the CRB *in vitro* study from the EF5 *in vivo* binding by using antibodies specific for each drug (EF5: Elk 3-51; EF3: Elk-A8).

Tissue was available for CRB studies in 10 patients (plus two additional patients who did not receive EF5, Table 2b). Eight patients had head and neck tumors and the skin tissue was removed from the face/neck whereas in two patients with lower extremity sarcomas, skin was removed from the thigh/pelvis. Four patients (plus the two not receiving EF5) had CRB studies performed on normal skin; five patients had CRB

Table 2b. Summary of cube reference binding

	Whole cube	Dermis	Epidermis	Hair follicle*	Sebaceous gland*
Cube average value (SD)	996 (552)	596 (340)	1,148 (637)	798 (1,257)	389 (134)
% of average cube binding (SD)	—	56 (11)	111 (6)	95 (31)	69 (14)
Cube min	308	136	302	206	225
Cube max	2152	1167	2290	2790	538

SD, standard deviation.
The CRB is expressed in absolute fluorescence values. Note that only dermis and epidermis were seen in all cubes. Thus, the values for the skin appendages represent the average of only a subset of cubes*. The CRB range (cube max: cube min) is increased substantially by the low values seen for the extremity samples of the sarcoma patients (no. 91 and no. 92).

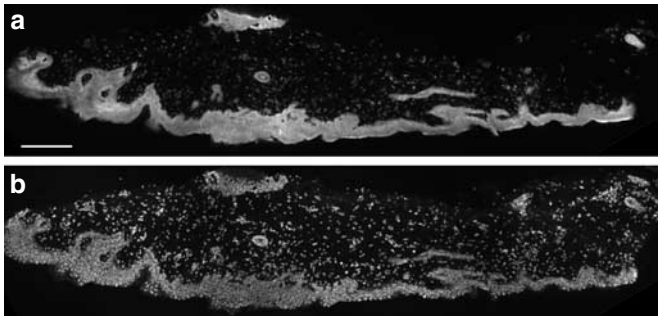

Figure 3. Tissue cubes for calculation of CRB. (**a**) Brightest tissue section of a tissue ''cube'' incubated *in vitro* under conditions of 0.2% pO_2 in the presence of EF3 (bar = 0.25 mm). (**b**) The corresponding Hoechst 33342 image, demonstrating the locations of nuclei. The fluorecence values obtained from the analysis of (**a**) represent the maximum binding that this specific tissue in this specific patient is capable of, for example, the CRB. CRB is used as the denominator and *in situ* binding is used for the numerator in the calculation of % maximum EF5 binding. We consistently found that dermis CRB was lower (about two-fold) than in the other skin substructures, even taking into account the lower cellular density in the dermis.

studies performed on irradiated skin and one patient had both irradiated and nonirradiated skin CRB studies performed. The time interval between radiation and the EF5 study was 1 and 3 months for the patients having surgery in the pelvic/thigh area, compared to 4, 8.5, and 10 months for the patients having surgery in the head and neck region.

EF3 binding values from the CRB studies are reported on a scale of absolute fluorescence. In all tissue cubes photographed and analyzed, a portion of the epidermis and dermis was included. However, glandular tissue or hair follicles were identified in only four tissue cubes from irradiated tissue and three cubes from nonirradiated tissue. An example of an ''optimal'' CRB study is shown in Figure 3. This image shows that cells throughout the skin bound EF3 at similar rates (about a factor of 2 lower in the dermis). Despite the consistent observation of similar maximal binding within the various substructures of the skin in a cube from a single patient, substantial differences in this end point were observed between patients (Table 2b).

There was no significant difference in the overall CRB values of irradiated ($n = 7$, average = 954, SD = 342) and nonirradiated ($n = 6$, average = 1,045, SD = 732) skin tissues. Whereas most of the tissues were from the head and neck region, two samples of irradiated tissue were obtained from patients with lower extremity sarcomas. The tissue samples from the lower extremity had substantially lower CRB compared to the tissues from the head/neck region (average = 335, SD = 27 *vs.* average = 1,116, SD = 525, respectively). While this difference is highly statistically significant, there are too few samples from the lower extremity for a valid conclusion at this time. Thus, inclusion of the data from the two lower extremity samples caused a much higher apparent range of CRB values (variation by a factor < 4 *vs.* > 7 with their exclusion *vs.* inclusion, respectively). This large difference between skin CRB in patients with extremity sarcomas versus head and neck tumors is discussed with respect to other patient variables, below. However, since EF5 binding analysis on all skin appendages were not available in all samples nor even in all patients, a direct comparison of their means and standard deviations cannot be made. The values are reported with this caveat, in Table 2c.

Non-oxygen dependent metabolism of drug. While the CRB results showed that maximum binding was similar among the skin substructures of each patient, it was necessary to demonstrate that this binding was hypoxia-dependent. Using skin tissue from a patient who did not receive EF5, cubes were incubated with EF5 *in vitro* using gas phase oxygen concentrations of either 0.2 or 95% (Koch, 2001). For the latter situation, binding was decreased more than 30-fold (data not shown; image was completely black) compared with the former, irrespective of skin substructure considered. Therefore, all EF5 binding in skin appears to be hypoxia-dependent.

EF5 binding is dependent not only on the inverse of oxygen concentration but also on drug concentration multiplied by time. To account for drug concentration in the calculation of *in situ* tissue binding, we measured EF5 in the patient's blood 1 hour after the drug was given and again when the tumor was removed. It was then possible to integrate the values of EF5 blood concentration over time to calculate each patient's EF5 drug exposure, known as the area under the curve (AUC). As reported in the EF5 Phase I trial (Koch *et al.*, 2001), the drug AUC varies by about a total factor of 2 for the patients described herein (Table 2a; range

Table 2c. Summary of *in situ* EF5 values corrected for CRB

	CF_{95} values for skin and its appendages			
	Dermis	**Epidermis**	**Hair follicle**	**Sebaceous gland**
In situ average value (SD)	0.2 (0.4)	6.9 (4.7)	27.5 (13.9)	13.6 (13.1)
In situ minimum	0.00	1.2	6.0	4.0
In situ maximum	1.4	17.7	46.0	38.9
Term to explain ranges of tissue pO_2	Physiologic pO_2	Physiologic to moderate hypoxia	Modest to severe hypoxia	Modest to severe hypoxia

CF, cumulative frequency; CRB, cube reference binding; SD, standard deviation; PO_2, oxygen partial pressure.
The value provided (CF_{95}) is defined such that 95% of the image pixels exist at or below the stated percentage of maximal binding (corrected for all parameters). In cases where a CRB value was not available from the same patient, an average value was used. Using the average relationship between EF5 binding and pO_2 determined in *in vitro* studies (Table 2d) and assuming that the same relationship holds for human skin, one can assign oxygen ranges to the skin and its appendages. Thus, oxygen levels in the dermis are consistently in the physiological range, while those of the epidermis range from physiological to moderately hypoxic. Oxygen levels for both sebaceous glands and hair follicles vary from modest to severe hypoxia.

Table 2d. Relationship of EF5 binding and tissue pO_2

Description	**PO_2 (%)**	**PO_2 (mm Hg)**	**% Maximal binding (CRB)**
Physiologic PO_2	10	76	1
Modest hypoxia	2.5	19	3
Moderate hypoxia	0.5	3.8	10
Severe hypoxia	0.1	0.76	30
Anoxia	0.0	0.0	100

CRB, cube reference binding; PO_2, oxygen partial pressure.
In this table, terms used to describe tissue oxygenation have been approximated by data from *in vitro* experiments (Koch, 2001).

1.2–2.4, average 1.28 ± 0.29 mM hours). Of course, drug AUC only accounted for patient to patient variation and was constant with the various skin substructures in an individual patient.

Accounting for CRB and AUC, the corrected relative intensities for the various skin substructures are summarized in Table 2d. These data show that dermal oxygen levels were consistently in the physiological range while those of the epidermis ranged from physiological (oxic) to moderately hypoxic (Table 2d). Oxygen levels for both sebaceous glands and hair follicles varied from modest to severe hypoxia. EF5 binding values and gradients were consistent with the pattern of blood vessels (anti-CD31 antibodies) and proliferating cells (anti-Ki67 antibodies), which were located primarily in the dermis (Figure 4).

Similarities in the rates of *in situ* binding between skin substructures within the same patient suggest minimal intra-patient variability in tissue nitroreductase activity. Conversely, the substantial differences in the rates of *in situ* binding between patients suggest that these variations were related to corresponding differences in nitroreductase levels. We considered several factors including site of tissue collection, patient age and gender that might explain such differences. The site of tissue collection (CRB in skin from head/neck region > skin from lower extremity) was the only factor that was associated with the CRB value. Therefore, we also analyzed the relationship between the absolute values of CRB of tumor tissue *versus* the CRB of skin removed at the surgery site. The specific question being posed was whether each patient had a characteristic ''whole body'' nitroreductase activity, irrespective of tissue site. A statistically significant correlation was found between the tumor and skin CRB (Figure 5). These data support the hypothesis that each patient may have a ''characteristic'' level of cellular nitroreductases. Note that the two lowest values in this graph represent the two skin tissue samples from patients with extremity sarcomas. Owing to the small patient numbers, we do not know the heterogeneity, etiology, or significance of the differences in CRB values/nitroreductase levels between patients with sarcomas *versus* head and neck cancer. Further insight will only come from enrolling more patients in these studies.

DISCUSSION

Despite many years of study, considerable uncertainty regarding the level(s) of skin oxygenation has remained. Evidence from rodent studies supports the presence of skin hypoxia because its radiation response could be increased by drugs known to act as oxygen-mimetic radiosensitizers (Stewart *et al.*, 1982; Rojas *et al.*, 1992; Stevens *et al.*, 1995). Most attempts to confirm this finding were based upon the binding of prototypic hypoxia-binding drugs such as misonidazole. (Cobb *et al.*, 1989, 1990b, c) found a correlation between misonidazole retention in stratified squamous epithelia and elevated oxygen-independent reductase levels. They concluded that misonidazole binding in skin and other normal tissues (e.g. esophagus) was more likely due to high reductase levels rather than the presence of hypoxia.

In contrast, Parliament *et al.* (1992) found that binding of misonidazole to mouse esophageal mucosa was substantially inhibited when animals breathed 100% oxygen. Unfortunately, in the absence of a system to calibrate misonidazole binding, the question of whether the murine esophageal mucosa was hypoxic, and to what degree, remained unclear. It is important to note that ''nitroreductases'' are not enzymes whose function is to reduce nitro-compounds. Rather, they are enzymes that functionally reduce such drugs, and their actual identity is uncertain. EF5 was originally developed as an alternative to misonidazole because misonidazole was found to have a wide variation and poor characterization of

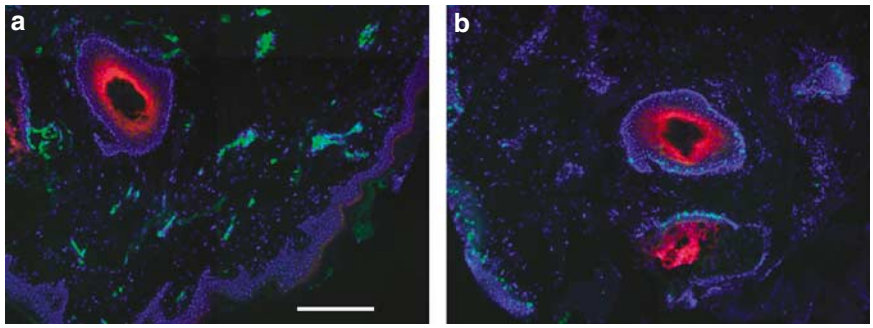

Figure 4. Co-localization of EF5 with proliferation and blood vessels. Images of skin from one patient presented to show the relationship between EF5 binding (red) and blood vessels (CD31; green (**a**)) or proliferating cells (Ki67; green (**b**)). In both panels, nuclei are counter-stained with Hoescht 33342 (blue; bar = 0.25 mm). The distribution of blood vessels supports the conclusion that the hypoxic regions are due to diffusion-limited oxygen metabolism since the spatial relationship between blood vessels and hypoxia is inverse. Furthermore, the distance between hypoxic regions and blood vessels is compatible with the range of oxygen diffusion suggested by Thomlinson and Gray (1955) (150–300 μ). Note that the proliferating cells (**b**) are in physiologically oxic to modestly hypoxic regions, explaining the sensitivity of these cells to radiation.

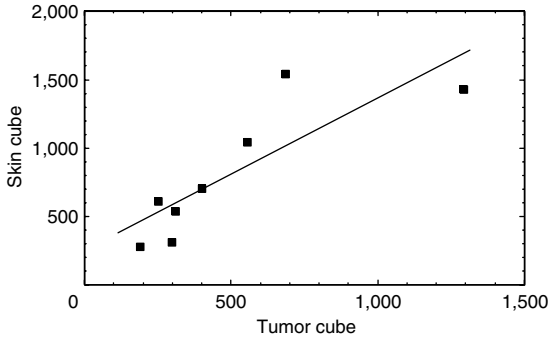

Figure 5. Relationship between CRB (absolute values) in tumor tissue *versus* overlying skin from each of eight patients ($r^2 = 0.7$, $P < 0.01$). These data were generated in order to determine whether the interpatient variation seen for CRB in skin would also apply to an unrelated tissue (tumor) from the same patient (i.e. does each patient have a characteristic nitroreductase activity?). The highly significant relationship found suggests that this question might be answered in the affirmative. However, the two lowest values are from patients with extremity sarcomas; hence, their skin samples were also taken from these anatomic sites, compared with most other values from the head and neck region. Owing to the small patient numbers, further insight into this question can only come from the accrual of more patients.

its effective nitroreductase levels (Franko *et al.*, 1987). Misonidazole has also been found to have both oxygen-dependent and oxygen-independent modes of metabolism (Koch *et al.*, 1993). Although comparative tests have shown no oxygen-independent metabolism of EF5 (Koch, 1990), our CRB data showed that nitroreductase activities vary substantially between patients, even with EF5. There are no data as to whether this might be true for inbred rodents. However, in the case of humans, it emphasizes the need to consider an individual patient's characteristics.

Our ability to use EF5 to measure skin oxygenation in patients undergoing tumor surgery provides a unique opportunity to apply calibrated and validated methods previously applied to tumors and some normal tissues (Shapiro *et al.*, 1997; Clyman *et al.*, 1999). Our data provide a consistent explanation for (1) the known radiation sensitivity of skin, since most dividing cells were found in regions with the least EF5 binding and (2) previous radiation sensitizer data, since portions of skin certainly exist at oxygen levels below venous pO_2 (~6% oxygen). This may be of substantial importance since hypoxia inhibits cell division and skin stem cells in the hair follicle outer root sheath bulb do not divide unless stimulated (Tumbar *et al.*, 2004).

Our studies also showed the presence of moderate to very high binding in some portions of human epidermis, sebaceous glands and hair follicles. These data partially agree with prior murine skin studies using tritiated misonidazole binding (Cobb *et al.*, 1990c), with the major difference being the frequency and heterogeneity of binding in human skin. In view of patient-to-patient and tissue-to-tissue variability in CRB, it is easy to understand the previous conclusion that such binding was an artifact of nitroreductase level variation. The difficulty with prior studies is that calibration studies on the rates of hypoxia binding were not calculated in individual patients or animals.

In human skin where the scale of substructures is larger, we can explain EF5 binding gradients by true tissue pO_2 gradients, supported by the distribution of blood vessels and proliferating cells. The presence and extent of these gradients are well documented in the cancer literature because of the importance of hypoxia in tumor resistance to therapy. The most common type of hypoxia gradient seen in tumors is diffusion-limited, which is the same as the gradients we see in skin. Our extensive studies of EF5 in cells and in tumors demonstrate that these gradients are oxygen dependent (Koch, 2001) and are not due to variations in nitroimidazole activity. Nevertheless, the heterogeneity and rarity of such gradients in skin beg the question of their etiology. While the very general finding of moderately high binding in the epidermis can be explained by oxygen diffusion from dermal blood vessels, the occasional high binding in hair follicles and sebaceous glands likely reflects local perturbations in oxygen demand. Oxygen delivery to the epidermis has previously been considered to arise from the dual planes of the skin surface (air) and dermis (blood vessels). Therefore, it is unexpected that the maximal *in situ* binding occurred in

the most superficial level of viable epidermal cells. Several explanations can be proposed. This may reflect relatively low oxygen supply from the air, or there may be oxygen consumption in the cornified layers and these cells could be without nitroreductase activity. Since EF5 binding is a metabolic process, one also has to consider the temperature profile throughout the tissue. Another possibility is that the oxygen diffusion constant may be different between subregions of the epidermis and dermis. These possibilities must be tested in further studies.

We have no means to measure the CRB of the individual hair follicles and sebaceous glands with high *in situ* binding, and we cannot formally exclude localized variations in nitroreductase activity. However, we can provide an estimate of the magnitude of such variations, since *in situ* EF5 binding is measured on an absolute fluorescence scale. The absolute EF5-derived fluorescence in high binding follicles, sebaceous glands and epidermis is 20- to 50-fold higher than in other low-fluorescence regions. It is very unlikely that such variations represent local variations in nitroreductase activity particularly since the high-binding areas are consistently found at a distance from the nearest blood vessels. Additionally, the absolute values *in situ* are not out of the range (i.e. not greater than) predicted by the CRB. For similar tissue appendages throughout the cube, we have not yet seen heterogeneous local variations in binding.

Considerable controversy remains regarding nitroimidazole binding in squamous cell-containing tissues. ''Nonphysiologic binding'' has been reported in squamous cell tumors removed from patients who received the hypoxia-detection marker pimonidazole (Janssen *et al.*, 2004). These tissues were stained with both anti-pimonidazole antibodies and nitro-blue tetrazolium, which is reduced by ''nitroreductases'' to a blue color. Pimonidazole staining was seen in 56% of keratinized areas and of these, 78% showed increased nitro-blue tetrazolium staining. These values indicated that high reductase levels were not always required for pimonidazole staining. However, it may not be the case that the same enzymes involved in reduction of pimonidazole are involved in the reduction of nitro-blue tetrazolium (Cobb *et al.*, 1990a; Janssen *et al.*, 2004). Certainly, one could not conclude that the oxygen dependent kinetics of such enzymes is independent of the drug used.

We have taken a different approach to examine whether EF5 binding in keratinized regions of the skin is oxygen-dependent. Rather than trying to assess the reductases by using an alternative and uncharacterized agent such as nitro-blue tetrazolium, we have used the binding of our well-validated oxygen markers EF5 or EF3 as the indicators. Our approach, previously reported with tumor tissues (Evans *et al.*, 2000, 2001a, 2004a, 2006), allows us to probe the ability of the specific tissue and its associated enzymes to reduce EF5, even if the exact enzyme is not known. To summarize these ''calibration'' studies, internal variations in nitroreductase activity, as exemplified by the CRB data, suggest the presence of only a small tissue-to-tissue difference for an individual patient, with dermis being about two-fold less than epidermis and its appendages. We can conclude that the large differences in *in situ* skin binding must be directly caused by substantial oxygen gradients.

Our data do not support the presence of radiation-induced hypoxia in skin. However the number of patients studied was small and there was substantial heterogeneity regarding the radiation doses received and the time between radiation therapy and tissue examination. The questions of whether hypoxia is a consequence of irradiation is still debated in the literature (Denham and Hauer-Jensen, 2002). Rudolph *et al.* (1994) reported the absence of ischemic lesions in irradiated skin and observed that transcutaneous pO_2 remained normal in a series of 100 patients affected by radiation-induced skin damage. Similarly, Aitasalo and Aro (1986) reported only a transient change in oxygenation after radiation in a rabbit model.

Hypoxia has been reported to be important in tissue biology and pathology. Hypoxia is a well-described factor in both radiation (Brown and Lemmon, 1991) and chemotherapy resistance (Adams, 1981). In the last decade, molecular signaling via the HIF pathway has received a great deal of attention regarding hypoxia's role in tumor aggression (Brahimi-Horn *et al.*, 2001). Of particular relevance to the skin is the recent observation that the presumed hypoxic microenvironment of the skin could contribute to Akt-mediated melanocyte transformation and may mediate the development of melanomas (Bedogni *et al.*, 2005). Tissue pO_2 is intimately involved with non-neoplastic tissue pathologies such as stroke and myocardial infarction (Acker and Acker, 2004) as well as mediating normal tissue repair and wound healing (Hunt *et al.*, 2004; Tandara and Mustoe, 2004). These processes have also been associated with oxygen-related signaling cascades such as the generation of reactive oxygen species and/or nitric oxide (e.g. see Rodrigo *et al.*, 2005). Oxygen levels are also thought to maintain normal function in tissues such as the retina (Yu and Cringle, 2005). Thus, the work presented herein is of very general interest since EF5 will measure hypoxia of almost any etiology and in any tissue type. As emphasized in this paper, however, in order to quantitatively evaluate tissue pO_2 several specific control studies must be performed.

We conclude that the skin dermis is well-oxygenated and associated with the presence of numerous blood vessels. Proliferating cells are in these regions; their damage by radiation explains acute radiation ''dermatitis''. In contrast, substructures of the human skin may contain regions of hypoxia; the etiology of the location and patterns of these regions, as well as their biologic and clinical significance requires further study. The techniques described for analyzing EF5 binding are generally applicable to almost all tissue systems.

MATERIALS AND METHODS

Human subjects

This study was conducted according to the Declaration of Helsinki Principles. Written informed consent, approved by the University of Pennsylvania Institutional Review Board which reviews ethical issues, Clinical Trials and Scientific Monitoring Committee of the University of Pennsylvania Cancer Center, Cancer Therapeutics

Evaluation Program and the National Cancer Institute was obtained from all patients entered on this study.

EF5 studies

Drug administration: The National Cancer Institute, Division of Cancer Treatment (National Cancer Institute/DCT) supplied EF5 in 100 ml vials containing 3 mg/ml EF5 in water with 2.4% alcohol and 5% dextrose. Based on a previously conducted Phase I trial, the drug solution was administered intravenously via a peripheral catheter at a rate of approximately 350 ml/hours to a total dose of 21 mg/kg (Koch *et al.*, 2001). Eight to 10 ml of blood was collected in a heparin tube preceding drug injection 1 hour after the start of drug injection and at the time of surgery. The EF5 content was determined in plasma using HPLC, as previously reported (Koch *et al.*, 2001). Based on these values, the drug AUC was calculated for each patient. As EF5 binding is inversely proportional to tissue pO2 and directly proportional to drug exposure levels, it is important that tissue EF5 binding values be corrected for the AUC (Koch *et al.*, 2001; Evans *et al.*, 2006).

Tissue acquisition, staining, photography, and analysis: Approximately 24 hours following completion of drug administration, the patient was taken to surgery. The skin tissue studied herein was removed as part of the procedure required for tumor resection and/or reconstructive surgery. In patients where surgery included incision into previously irradiated tissue, irradiated skin was sampled and studied. In one patient, it was possible to collect both unirradiated and irradiated skin. Skin tissue was also obtained from two patients who did not receive EF5 preoperatively but were under going tumor excision (negative control). Tumor tissue was collected in all patients.

Skin tissue from each patient was frozen for analysis of *in situ* EF5 binding. Whenever possible, the skin was oriented during freezing to ensure that a cross-section of the entire skin could be obtained. Frozen sections (10 μm thickness) were cut using a Microm HM 505 cryostat (Carl Zeiss, MICROM Laborgeräte GmbH, Waldorf, Germany) then fixed, blocked and stained with Cy3-conjugated anti-EF5 antibodies (ELK3-51 produced in-house), as previously reported for tumors (Evans *et al.*, 2004a). This staining sample is referred to as the ''Regular Stain''. A representative section from each patient was also stained with the standard anti-EF5 antibody mixed with an excess of authentic EF5 drug, 0.5 mM. This control is termed ''Competed Stain''. The excess drug binds to all specific sites on the antibody and prevents specific binding to tissue adducts. The Competed Stain values were subtracted from the absolute fluorescence values for the Regular Stain sections. A no-stain control was also performed.

Following staining, sections were stored at 4°C in phosphate-buffered saline containing 1% paraformaldehyde, a condition that stabilizes the antibodies and prevents fluorescence loss (Koch, 2001). Images of the sections were made using a cooled CCD camera and Nikon LabPhot fluorescence microscope (Nikon Instrument Group, Inc., Melville, NY, USA) as described previously (Evans *et al.*, 2004a). This system generates images where fluorescence can be assessed on an absolute scale by accounting for the microscope's lamp intensity and camera exposure time (Koch, 2001). Since EF5 binding is also directly proportional to drug concentration and time, the fluorescent signal obtained from the tissue sections was normalized for the patient's drug AUC (Koch *et al.*, 2001). The AUC is determined from HPLC analysis of plasma EF5 concentration at the time of drug injection and at the time of surgery (Koch *et al.*, 2001). We have previously shown that the fluorescence signal is a direct measure of absolute EF5 binding (Koch, 2001).

Finally, to directly compare binding from one tissue to the next, corrections must be made for the inherent EF5 metabolic rate. We have shown that the oxygen dependence of EF5 binding is consistent among many cell types *in vitro,* even though the absolute binding level may vary. With the knowledge of the maximum binding rate that the tissue is capable of, it is possible to determine the *in situ* binding of the tissue on a pixel-by-pixel basis. Maximum binding, rather than minimum, is determined in order to optimize accuracy at low oxygen levels. The maximum binding rate is referred to as CRB and is described below (Koch, 2001; Evans *et al.*, 2004a). Thus, the end points for EF5 binding presented herein reflects both the level and proportional area of binding (Laughlin *et al.*, 1996; Wilson *et al.*, 2000; Evans *et al.*, 2004a).

Data were summarized by providing a cumulative frequency analysis of all pixels in an image. Selected points on the cumulative frequency curve were denoted by $CF_{\#\#}$. Thus, $CF_{95} = 20$ means that 95% of the EF5 values in the image were at or below 20% of the CRB. Following this nomenclature, median EF5 binding is denoted as CF_{50} (Evans *et al.*, 2004a). We have previously described our procedure to produce oxygen maps from the EF5 binding images (Koch, 2001; Evans *et al.*, 2004a). The accurate generation of such oxygen maps requires that the oxygen dependence of EF5 binding is known. Based on current data for tumor, fibroblast and other cell lines, we have defined several ranges of tissue pO_2 and their approximate equivalents of percent maximum EF5 binding (Koch, 2001). Thus, the qualitative terms physiological, modest hypoxia, moderate hypoxia, severe hypoxia, and anoxia have been assigned average oxygen partial pressures as summarized in Table 2d.

In about half of the patients studied, sufficient skin tissue was available to determine CRB, as well as *in situ* binding (Koch, 2001; Evans *et al.*, 2004a). Briefly, for CRB determination, 2 mm skin cubes were cut from the main tissue sample with surgical blades. Each cube had the epidermis intact. These cubes were incubated under hypoxic conditions (0.2% oxygen) with 200 μM EF3 (a drug closely related to EF5) for 3 hours. The EF5 dose/time interval was chosen to approximate that of the *in situ* tissue exposure. The tissue cubes were incubated at 0.2% oxygen rather than 0% oxygen in order to minimize binding in the damaged cells at the edge of the cube (due to artifacts of cutting the cube). We count on tissue respiration to allow the maximum binding at close to 0% pO_2 to be a few cell layers inside the cube surface. Depending upon the cell density (which is low in the dermis), tissue damage or variations in the distance of the cells of interest to the surface of the ''cube'', there can be substantial variations in binding, but we only consider its regionally maximal value.

Cubes were frozen, sectioned on the cryostat (10 μ), stained with the appropriate monoclonal antibody and imaged. EF3 and EF5 have essentially equivalent binding characteristics (Koch, 2001). Owing to the difference in their sidechain structure, it is possible to stain the tissue cubes with a distinct Cy3-conjugated antibody, Elk5-A8, with little interference from EF5 adducts that may be present from the *in situ* binding (Koch *et al.*, 1995; Busch *et al.*, 2000). Since there was heterogeneity of cell types and cell densities in skin, subanalyses

of the *in situ* EF5 binding and *in vitro* EF3 binding (CRB) were performed. Analysis of the level of EF5 binding in each photomicrograph was performed by identifying each of the anatomic substructures present in each section: dermis, epidermis, sebaceous glands, and hair follicles. It should be noted that not every section contained all structures (a particular problem for the limited tissue available from cubes). Calculation of the absolute binding (cubes) or CF_{95} (*in situ* tissues) was then performed on each region of each tissue section. In order to analyze all areas, a tissue mask was made by counterstaining sections with Hoechst 33342 to identify nuclei. This image was then translated in MATLAB into the tissue mask (Evans *et al.*, 2004a). This method successfully eliminates acellular areas such as hair shafts and sebaceous secretions. However, this masking process may slightly underestimate the degree of dermis binding, which has a substantially lower cell density than epidermis and appendages (no binding would be expected, nor was any found, in extracellular matrix or other acellular regions). A subset of tissue sections was also stained for Ki67 (proliferation) and/or CD31 (blood vessels) as previously reported (Evans *et al.*, 2001b).

Histopathological analyses

The anatomic correlation of the EF5 binding regions was made by counterstaining the EF5-stained slides with hematoxalin and eosin. A subset of hematoxalin and eosin slides was reviewed by the pathologist (PZ) to identify the characteristics of sebaceous glands and hair follicles. This information was then applied to analyses on the other tissue sections.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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