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Spatiotemporal assessments of dermal hyperemia enable accurate prediction of experimental cutaneous carcinogenesis as well as chemopreventive activity

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Abstract

Field cancerization refers to areas of grossly normal epithelium that exhibit increased risk for tumor occurrence. Unfortunately, elucidation of the locoregional changes that contribute to increased tumor risk is difficult due to the inability to visualize the field. In this study, we use a non-invasive optical-based imaging approach to detail spatiotemporal changes in subclinical hyperemia that occur during experimental cutaneous carcinogenesis. After acute inflammation from 10 weeks of ultraviolet B (UVB) irradiation subsides, small areas of focal hyperemia form and were seen to persist and expand long after cessation of UVB irradiation. We show that these persistent early hyperemic foci reliably predict sites of angiogenesis and overlying tumor formation. Over 96% of tumors (57 of 59) that developed following UVB or DMBA/PMA treatment developed in sites of preexisting hyperemic foci. Hyperemic foci were multifocal and heterogeneously distributed and represented a minor fraction of the carcinogen-treated skin surface (10.3% of the imaging area in vehicle treated animals). Finally, we also assessed the ability of the anti-inflammatory agent celecoxib to suppress hyperemia formation during photocarcinogenesis. The chemopreventive activity of celecoxib was shown to correlate with its ability to reduce the area of skin that exhibit these hyperemic foci, reducing the area of imaged skin containing hyperemic foci by 49.1%. Thus, we propose that a hyperemic switch can be exploited to visualize the cancerization field very early in the course of cutaneous carcinogenesis and provides insight into the chemopreventive activity of the anti-inflammatory agent celecoxib.

Keywords

Photocarcinogenesis; hyperemia; non-melanoma skin cancer; mesoscopic imaging; and chemoprevention

INTRODUCTION

Field cancerization describes the increased risk for additional tumor formation following the appearance of a first tumor within an area exposed to carcinogens (e.g. UVB) (1–3).

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However, the exact nature of the field involvement is unclear as the “field” generally cannot be visualized but is established post hoc after tumor(s) begin to appear. Recently, fiber-optic endoscope-based measurements of superficial hemoglobin (Hgb) content at multiple random sites have shown that a measurable early increase in blood supply (EIBS) is detected not only in the tumor stromal environment, but is also seen in the histologically normal mucosa of the GI tract in areas both near and remote from the neoplastic lesion (4, 5). Moreover, EIBS may occur very early during tumorigenesis, as EIBS is seen prior to tumor formation in the normal colonic mucosa of azoxymethane (AOM) treated rats compared with the colons of vehicle treated rats (6). The authors also demonstrated that the increased hyperemia represents increased angiogenesis (6). Thus, this group proposes that EIBS is a feature of field cancerization (6). Unfortunately, these studies use random point measurements taken with a probe-based optical sensor and are limited in that they fail to visualize EIBS across a carcinogenic field. Thus, it is unclear whether EIBS exhibits a direct correlation to sites in which tumors will eventually form or represents a homogenous change to the entire field at risk.

Studies utilizing painstaking microscopic examination of tissues have also indicated that angiogenesis can occur relatively early in tumor development (7). In this case, angiogenesis was associated with early hyperplastic changes that were multifocally or non-homogeneously distributed (7). Regrettably, the process of obtaining tissue samples for microscopic examination results in the inability to monitor these sites of angiogenesis and hyperplasia over time to verify their association with future malignancy. Thus, while this data suggests that hyperemia within the (pre)carcinogenic field is heterogeneously or multifocally distributed, the question remains as to whether these sites are spatially fixed over time. Given the inability to determine *a priori* the specific site where a tumor is likely to occur, microenvironmental changes necessary for tumor development could be missed or their significance underestimated if they are non-homogenous in distribution, represent a minor fraction of the available surface, or are not visible on clinical inspection. In contrast, changes seen over the whole field may be readily apparent, but their importance to tumorigenesis may be over-estimated. Thus, while we have experienced tremendous growth in our understanding of the microenvironment that surrounds visible tumors, we have a more limited understanding of the premalignant microenvironment due to the inability to target studies to specific sites of future tumor appearance within a cancerization field (8).

The idea that angiogenesis is turned on early during carcinogenesis is supported by studies in human tumors and in mouse models of multi-stage chemical carcinogenesis (7, 9–11). While the mechanisms for this early induction of angiogenesis during tumorigenesis are incompletely understood, existing tumors are known to induce angiogenesis by increased production of pro-angiogenic inflammatory cytokines/chemokines (inflammatory angiogenesis) (10, 12). Moreover, the majority of human cancers are thought to be derived from environmental exposures and lifestyle choices (13–15). Importantly, a common feature of these environmental and lifestyle choices is that they promote inflammatory angiogenesis (10, 14, 16, 17). The importance of inflammatory angiogenesis in early stages of tumorigenesis may also be inferred by the ability of a number of chemopreventive agents, such as the anti-inflammatory cyclooxygenase-2 (COX-2) inhibitors, to suppress both the inflammation and angiogenesis that are observed following the application of carcinogenic insults (10, 14, 16, 17).

In this study, we utilize a novel technique that couples optical measurement of Hgb content to a non-invasive imaging platform in live animals to determine detailed spatiotemporal patterns of hyperemia formation in a large area ($15 \times 45 \text{ mm}^2$) during the course of cutaneous chemical and photocarcinogenesis. We then correlate the areas of hyperemia with subsequent tumor formation to verify that this methodology visualizes the (pre)carcinogenic

field. Given the known ability of celecoxib to act as an anti-inflammatory and anti-angiogenic chemopreventive agent, we also examine the effects of celecoxib on the spatiotemporal extent of hyperemia formation. We propose that this new approach to visualize EIBS provides a novel method to non-invasively visualize field cancerization early in the course of cutaneous carcinogenesis.

MATERIALS AND METHODS

Chemical carcinogenesis study

8 female FVB/n mice were treated with DMBA/PMA as previously described (18). After shaving and depilatory cream application to remove hair from the imaging area, the mice were imaged and Hgb content assessed as described (18).

Photocarcinogenesis studies

SKH-1 hairless albino mice (Charles River Laboratories, Wilmington, MA, USA) were irradiated with one minimal erythema dose of UVB (2240 J/m²) three times per week (M,W,F) as previously described (19). Studies by us and others have shown that this treatment consistently results in initial tumor formation within 11–12 weeks of treatment (19, 20). We therefore discontinued UVB treatments after 10 weeks of treatment. This resulted in a cumulative UVB dose of 67.2 kJ/m², which exceeds a known carcinogenic cumulative UVB dose of 26.2 kJ/m² (21). For celecoxib studies (20), a chemopreventive dose of 0.5 mg of celecoxib (LC Laboratories, Woburn, MA, USA) in 0.2 ml acetone (or vehicle alone) was applied topically immediately after each UVB irradiation and 3 times per week after discontinuation of the UVB irradiations. We imaged the irradiated mouse skin every 2 weeks, using our microvascular imaging platform (22, 23) (Supplemental Methods and Figs S1 – S3 for detailed performance characteristics). To obtain sequential images from identical areas over time, we had reference tattoos placed on each mouse to form a rectangular imaging grid. The mice were lightly sedated using ketamine/xylazine for immobilization during imaging.

Histopathologic assessment for neoplastic lesions

Visible tumors were scored on a weekly basis when exophytic growths exceeding 1 mm in diameter were observed. At the end of the study (20 weeks after discontinuing UVB treatments), all visible tumors were removed and formalin-fixed for tumor classification by a dermatopathologist. In addition, skin from areas of high and low Hgb content that did not contain visibly apparent tumors were removed and were formalin-fixed. Paraffin-embedded sections were stained with hematoxylin & eosin (H&E) and examined by dermatopathologists for the presence of small tumors not visible macroscopically. Tumors were classified as previously described ((19) and Supplemental Methods).

Microvascular density (MVD)

Following heat-induced antigen retrieval in citrate buffer, pH 6.0, formalin-fixed paraffin-embedded tissue slides were subjected to immunolabeling using rat monoclonal anti-mouse CD31 antibodies (Clone SZ31, Dianova, Hamburg, Germany). Stained sections were blinded and 5 random 400× fields per tissue section were counted by three different individuals. Microvessels were only counted where a visible lumen was observed. The mean value derived from the MVD counts from all three individuals was used for data presentation and statistical analysis.

Statistical analysis

Non-parametric and parametric statistical analysis was used where appropriate (Supplemental Methods). Statistical significance was assigned at a p -value < 0.05.

RESULTS

Imaging of microvascular Hgb content during chemical carcinogenesis and photocarcinogenesis demonstrates that EIBS precedes visible tumor occurrence

We first utilized a DMBA/PMA chemical carcinogenesis protocol to examine the spatiotemporal changes in Hgb content during cutaneous carcinogenesis (18). As expected, DMBA/PMA treated mice developed tumors within 10 weeks of initiating treatment, eventually reaching approximately 2 tumors per mouse (Fig 1A). Nine durable tumors occurred within the $12 \times 12 \text{ mm}^2$ imaging area. Overall averaged Hgb content within the imaging area was unchanged in the DMBA/PMA treated mice relative to vehicle control mice prior to 10 weeks, but was increased in the DMBA/PMA treated mice beginning in week 10 (Fig 1B). Although the increased Hgb content correlates with increasing tumor burden (right panel, Fig 1C), we noted that all 9 tumors were seen to develop in focal areas of hyperemia that were apparent prior to the visible appearance of tumors. This indicates that hyperemia may represent an early step in tumorigenesis. We next mapped out progressively smaller areas of increased Hgb content by using sequentially higher Hgb cut-offs for threshold mapping (Fig 1C). We found that all tumors occurred in preexisting areas of Hgb content > 1.6 mg/ml (20.4% of the imaged area). These observations suggest that our imaging methodology was measuring EIBS during early tumorigenesis. Unfortunately, the DMBA/PMA model had several deficiencies. First, the need for shaving and the use of depilatory creams made imaging difficult and could introduce hyperemia artifacts through mechanical or chemical irritation. Second, regional hair follicle cycling could contribute to some of the hyperemic response as angiogenesis is activated during the anagen phase of hair follicle development (24). Third, PMA is known to be an inducer of acute inflammation. Thus, we felt that this model had too many confounding variables for adequate analysis of EIBS.

UVB exposure represents the primary etiological agent for NMSC formation (25). We therefore switched to a mouse model of photocarcinogenesis using hairless, albino SKH-1 mice (26). SKH-1 mice exhibit a defect in hair cycling in which the hair follicles permanently arrest in catagen phase (26). Thus, the permanent hair loss makes this mouse more suitable for both imaging and UVB-irradiation studies, while the lack of hair cycling through anagen provides a better model for studies on dermal angiogenesis. Fig 2A illustrates a typical regional pattern of dermal Hgb content in non-UVB treated SKH-1 mice. The regional variability was marked by gradual changes in Hgb content, with higher levels seen overlying the spinal hump, and generally showing a symmetrical pattern on either side of the spinal midline.

For our UVB studies, we also wished to avoid the influence of UVB-induced acute inflammation (sunburn); thus, a cumulative carcinogenic dose of UVB was administered by repetitive UVB irradiations over the first 10 weeks and then discontinued (21). Examining Hgb content beginning two weeks after cessation of UVB dosing allowed us to examine changes in blood supply at the time when tumors initially begin to appear in this mouse model (19, 20), but after the acute sunburn effect had resolved. This also mimics human behavior, in which sun-avoidance strategies are often employed only after significant cutaneous photodamage is apparent or a first tumor is found.

In Fig 2B, we show that 10 weeks of UVB treatment resulted in grossly visible tumor formation starting 2 weeks after discontinuing UVB treatments. As expected, peak tumor

multiplicity was suppressed by 40% in the celecoxib treated mice. We also noted that tumor-associated hyperemia is easily visualized using our bioimaging approach as markedly elevated Hgb content with sharply demarcated borders that tend to outline the tumor margins (Fig 2C). Indeed, focal hyperemia associated with tumors was seen to resolve in tumors that spontaneously disappeared (Supplemental Fig S4). Importantly, as in the 9 tumors seen in the DMBA/PMA studies, all 25 visually apparent tumors that occurred in our photocarcinogenesis studies were preceded by focal areas of increased hyperemia (Fig 2C). These hyperemic foci either persisted after resolution of the acute UVB-induced erythema reaction, or began to appear within the early weeks after UVB cessation.

While the above studies showed that all visible tumors developed in smaller areas of the epidermis characterized by focal hyperemia, not all areas of hyperemia were seen to develop visibly apparent tumors. We therefore euthanized the mice 20 weeks after stopping UVB treatments and biopsied skin from areas of high and low Hgb content that did not contain a grossly visible tumor. After histopathologic examination of H&E stained sections, a number of papillomas and microinvasive squamous cell carcinomas (SCCs) were observed that were too small to be seen by visual inspection of the skin (Fig 2D and Supplemental Table 1). Of 34 microscopically observed tumors seen in irradiated vehicle or celecoxib treated skin, 32 tumors occurred in areas of high Hgb content. Thus, a total of 57 tumors (25 large visible tumors and 32 microscopic tumors) were seen in the much smaller areas of skin exhibiting hyperemia, while only 2 microscopically observed papillomas were seen in the much larger area of skin without hyperemia. Interestingly, celecoxib did not cause a significant change in the average numbers of microscopically observed tumors within the existing areas of hyperemia (Fig 2D).

Celecoxib's chemopreventive activity correlates with its ability to suppress the area of treated skin that exhibits focal hyperemia rather than a global suppression of Hgb content

In Fig 3A, we contrast the typical regional pattern of Hgb content in non-irradiated mouse skin with the Hgb content map of a mouse 6 weeks after stopping UVB treatments. Compared with the symmetrical and gradual changes in Hgb content noted in non-irradiated mice, mice treated with a carcinogenic dose of UVB showed a non-symmetrical distribution, with focal areas of high Hgb content exhibiting distinct borders and an abrupt transition to intense hyperemia. Importantly, areas surrounding these focal hyperemic areas often exhibited lower Hgb content than non-irradiated mice (more blue coloration). Based on this focal pattern of intense hyperemia, we felt that overall averaged Hgb content from the entire imaging failed to adequately assess either the Hgb distribution pattern or tumor risk. It was therefore not particularly surprising that there was actually a modest and significant decrease in overall Hgb content in UVB-treated skin relative to non-irradiated mice when the Hgb content was averaged over the entire imaging area (Supplemental Fig S5). Moreover, in celecoxib treated mice, overall Hgb content failed to show any correlation with celecoxib's chemopreventive activity (Fig S5).

Insofar as averaged Hgb over the entire imaging area did not appear to adequately assess the changes in hyperemia that we were observing in irradiated mice after cessation of UVB treatments, we therefore utilized a different analytical approach that took into account the highly focal and heterogeneous pattern of Hgb content distribution. We first prepared a threshold map of hyperemic areas that had Hgb content greater than 1.6 mg/ml similar to that seen in Fig 1C. When Hgb content was measured only in these smaller foci of intense hyperemia (Fig 3B), we now saw that UVB treatment did indeed increase the amount of Hgb present within the hyperemic foci. Thus, the data supported our conclusion that hyperemic areas that persist following cessation of UVB treatments represented focal areas of more intense hyperemia than that observed with normal regional blood flow.

Interestingly, as with the tumor data in Fig 2D, the Hgb content within these hyperemic foci failed to correlate with the chemopreventive activity of celecoxib (Fig 3B).

In the above studies, we show that tumors almost invariably occur in small focal areas of intense hyperemia. However, neither the intensity of Hgb content within these areas nor the tumor burden is altered by a chemopreventive dose of celecoxib. We therefore hypothesized that if celecoxib treatment does not reduce tumor risk within these foci, perhaps it reduces tumorigenesis by suppressing the total area of involved skin at risk for tumor formation. In Fig 3C, we show that celecoxib treatment results in a suppression of the total imaging area exhibiting hyperemic foci formation by 49.1% (areas with Hgb content > 1.6 mg/ml were 10.3% & 5.5% of the imaging area for vehicle treated and celecoxib treated animals, respectively). This reduction in the area at risk for tumor formation correlates well with celecoxib's chemopreventive activity ((Fig 2B) and (20, 21)) As expected based on the data in Fig 2D, we show an essentially identical rate of visible tumor formation within the hyperemic areas that persist in both vehicle treated and celecoxib-treated mice (0.22 and 0.21 tumors/mm²).

Areas of focal hyperemia not only persist in the absence of a visible tumor, but also expand following cessation of UVB treatment

As noted above, after discontinuing UVB treatments, areas of increased hyperemia were detected that persisted or formed over the ensuing weeks and months. Interestingly, an examination of Hgb content images revealed that these persistent areas of focal hyperemia appeared to expand over time (Fig 4A). To assess this quantitatively, we generated threshold maps of Hgb content > 1.6 mg/ml, measured the total area of skin exhibiting Hgb content above this threshold, and plotted the data at 2 week intervals after stopping UVB irradiations (Fig 4B). As expected, UVB irradiated mice treated with both vehicle and celecoxib showed an increase in the area of hyperemic foci over time. In addition, the expansion of hyperemic areas was seen to precede grossly observable tumor formation (Fig 4C). As in Fig 3C, celecoxib treatment suppressed the overall area of skin with high Hgb content at each imaging time point (Figs 4B & 4C).

Hyperemic foci correspond to areas of angiogenesis

In studies by others in rat colon, AOM-induced EIBS corresponded with increased angiogenesis (6). We therefore examined whether hyperemic foci exhibited evidence of angiogenesis. In Figs 5A–D, we show the histologic differences in skin sections obtained from non-tumor bearing areas of high and low Hgb content 20 weeks after stopping UVB treatments. Following H&E staining, we observed that areas of high Hgb content in UVB irradiated skin exhibited marked epidermal hyperplasia, a hypercellular dermis consistent with increased inflammatory cell infiltrates and increased numbers of small vessels (Figs 5D & E). In contrast, irradiated skin obtained from areas of low Hgb content were largely normal, with some focal areas of mild epidermal hyperplasia and a variable but generally mild increase in dermal hypercellularity (Fig 5B). In contrast, in non-irradiated control mice there was no apparent histologic change in areas of high and low regional blood flow (Figs 5A & C). To verify that focal hyperemia was associated with increased angiogenesis in irradiated mice, we assessed MVD. In Fig 5F, we show that MVD was significantly greater in areas of high Hgb content in UVB treated mice compared with areas of low Hgb content. Celecoxib treatment also had no significant effect on MVD in the smaller hyperemic areas that persisted in spite of celecoxib treatment. However, this was expected as we had already shown that celecoxib had no significant effect on Hgb content within hyperemic foci (Fig 3B).

UVB-induced acute erythema reactions (sunburn) are uninformative regarding future tumorigenesis or the chemopreventive efficacy of celecoxib

Our photocarcinogenesis studies were designed to remove potential interference from UVB-induced acute hyperemia. As we expected, a marked increase in Hgb content was observed using our imaging approach in UVB irradiated mice 72 hours after the final irradiation (upper left panel in Fig 6A). This increase in Hgb content was also visually apparent as an area of erythema (sunburn) in the photographic image (lower left panel in Fig 6A). Importantly, within a few weeks after discontinuing UVB treatments, visible erythema disappeared and was accompanied by a return to a largely normal pattern of regional Hgb content (right upper panel in Fig 6A). It is also important to note that transient UVB-induced hyperemia failed to correlate with sites of eventual tumor formation (Fig 6A; hashed circle). Nor did visible erythema reactions correlate with the chemopreventive activity of celecoxib. This subjective assessment was verified by measuring the acute UVB-induced erythema reaction using a narrow-band reflectance spectrophotometer (MexameterR, Fig 6B) (see Supplemental Methods).

DISCUSSION

Field cancerization is clearly present in human non-melanoma skin cancer, where field treatments are often necessary to treat multiple premalignant actinic lesions that occur on sun-damaged skin (27). Multiple theories have been proposed to explain how a cancerization field develops (3, 28). However, it is difficult to firmly establish the nature of early field development when the field cannot be visualized prior to grossly visible tumor formation. In our study, we provide multiple lines of evidence that hyperemia is an early marker of field cancerization and that our imaging approach visualizes the cancerization field in experimental cutaneous murine carcinogenesis: 1) Tumors almost invariably formed in areas of focal hyperemia that persist long after the cessation of carcinogenic UVB exposures. 2) Hyperemic areas in which tumors formed represented a minor fraction of the total treated area and were multifocal in nature. 3) The ability of celecoxib to suppress tumor formation correlated well with its ability to suppress the area of UVB-treated skin that exhibited high Hgb content. 4) Hyperemic foci not only persisted, but expanded in size long after cessation of UVB treatments. Moreover, expansion was seen to precede the appearance of a grossly visible tumor. 5) Hyperemic foci in areas where no tumor were present had characteristic early histologic changes associated with tumorigenesis, including epidermal hyperplasia, increased dermal hypercellularity suggestive of an inflammatory infiltrate, and increased angiogenesis. Importantly, these histologic changes were observed even though the tissue was removed long after UVB irradiations had stopped (20 weeks), indicating a mechanism for sustaining the epidermal hyperplasia and angiogenesis.

Our observation that both hyperemic foci and tumor risk are not homogeneously distributed, but rather are limited to a small heterogeneous segment of the entire carcinogen treated surface is important. Our methodology could potentially allow investigators to actually visualize this multifocal “field” to allow targeted biopsies to assess locoregional changes associated with early events in carcinogenesis, and could provide a method to assess the efficacy of chemopreventive agents. Moreover, the ability to target sites at high risk for tumor formation for longitudinal studies in live animals will provide important information regarding the early premalignant changes that are specific to the cancerization field. Our methodology would be of particular importance if validated in human studies, such as individuals with significant cutaneous photodamage or early premalignant actinic disease that are at high risk for non-melanoma skin cancer formation. Currently, studies in live volunteers are constrained by the limited amounts of tissue that can be ethically obtained. High variability due to sampling bias results in the need for high numbers of volunteers to

obtain statistically valid data or restricts studies to sites of visible premalignant or malignant lesions.

Studies of carcinogen-induced malignancy in skin and other tissues indicates that the angiogenic switch can be triggered much earlier during the carcinogenic sequence, occurring as early as the sustained hyperplastic response (7, 29). This differs somewhat from the classic model of tumor-induced angiogenesis, in which an existing avascular tumor switches on pro-angiogenic signals that provide the increased blood supply needed for tumor expansion and progression (7, 29). However, this classic model of an “angiogenic switch” is generally observed with the use of various oncogene-driven mouse models (29). Thus, our findings support the idea that the angiogenic switch occurs very early during cutaneous carcinogenesis rather than the classic model whereby the angiogenic switch occurs later in existing avascular visible tumors. However, further studies are necessary in our model to verify that angiogenesis is responsible for the hyperemic foci present prior to the time when tumors are evident.

One of the interesting findings of our study is that celecoxib’s chemopreventive activity correlates directly with its ability to decrease the total surface area exhibiting hyperemic foci. In contrast, celecoxib failed to suppress either tumor formation or the intensity of the hyperemia within these foci. A number of possible explanations could explain these observations, all of which would require further studies to verify. One hypothesis would be that the hyperemic foci represent patches of proliferating (pre)malignant keratinocytes that produce the pro-angiogenic cytokines necessary to trigger increased dermal blood supply. COX-2 inhibitors exhibit potent effects on the growth and survival of normal and neoplastic epidermal keratinocytes (30, 31). Thus, celecoxib treatment could limit the clonal expansion of these (pre)malignant epidermal patches, thus limiting their size. Alternatively, COX-2 inhibitors are known to suppress UVB-induced inflammation and this is closely associated with their anti-neoplastic activity (21, 26). Moreover, a persistent inflammatory stromal environment is associated with both angiogenesis and increased overlying epithelial tumor development (9, 10, 32). Thus, it is possible that celecoxib suppresses the formation of persistent inflammatory foci, which in turn suppress angiogenesis and overlying tumor development. In this case, it is unclear why some hyperemic foci are resistant to the effects of celecoxib. A trivial explanation would be that these areas failed to receive adequate levels of celecoxib due to variable topical delivery. However, this seems highly unlikely given that each mouse was treated on multiple occasions over the 30 week course of the study. Still, repeating these studies using systemic delivery of celecoxib would be of interest.

The risk of tumor development is well known to persist for years after the removal of carcinogenic insults. A number of theories have been advanced to explain these observations. This includes dormant tumors that are induced to progress due to an angiogenic switch that triggers increased blood supply (29) or the acquisition of proliferative self-sufficiency or survival advantage mediated by mutagenic events or a nurturing microenvironment (8, 33). Unfortunately, the inability to specifically identify tissue sites at the earliest stages of tumorigenesis limits the ability to define why risk for tumor development persists. Our data suggests that an unknown mechanism exists that sustains and actually expands areas of focal hyperemia after removal of a carcinogenic insult. As mentioned above, this could be due to continued clonal expansion of (pre)malignant patches or possibly the presence of inflammatory feedback loops driving persistent inflammatory angiogenesis. Identifying the engine that drives the persistence and expansion of these hyperemic zones could provide answers to how risk for tumor formation persists in individuals who undertake risk-avoidance behavioral changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*. 1953; 6:963–8. [PubMed: 13094644]
2. Dakubo G, Jakupciak J, Birch-Machin M, Parr R. Clinical implications and utility of field cancerization. *Cancer Cell International*. 2007; 7:2. [PubMed: 17362521]
3. van Oijen MGCT, Slootweg PJ. Oral field cancerization: Carcinogen-induced independent events or micrometastatic deposits? *Cancer Epidemiology Biomarkers & Prevention*. 2000; 9:249–56.
4. Wali RK, Roy HK, Kim YL, Liu Y, Koetsier JL, Kunte DP, et al. Increased microvascular blood content is an early event in colon carcinogenesis. *Gut*. 2005; 54:654–60. [PubMed: 15831911]
5. Gomes AJ, Roy HK, Turzhitsky V, Kim Y, Rogers JD, Ruderman S, et al. Rectal mucosal microvascular blood supply increase is associated with colonic neoplasia. *Clinical Cancer Research*. 2009; 15:3110–7. [PubMed: 19383816]
6. Tiwari AK, Crawford SE, Radosevich A, Wali RK, Stypula Y, Kunte DP, et al. Neo-angiogenesis and the premalignant micro-circulatory augmentation of early colon carcinogenesis. *Cancer Letters*. 2011; 306:205–13. [PubMed: 21493000]
7. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996; 86:353–64. [PubMed: 8756718]
8. Spencer SL, Gerety RA, Pienta KJ, Forrest S. Modeling somatic evolution in tumorigenesis. *Plos Computational Biology*. 2006; 2:939–47.
9. Hanahan D, Weinberg Robert A. Hallmarks of cancer: The next generation. *Cell*. 2011; 144:646–74. [PubMed: 21376230]
10. Albini A, Tosetti F, Benelli R, Noonan DM. Tumor inflammatory angiogenesis and its chemoprevention. *Cancer Research*. 2005; 65:10637–41. [PubMed: 16322203]
11. Abulafia O, Triest WE, Sherer DM. Angiogenesis in squamous cell carcinoma in situ and microinvasive carcinoma of the uterine cervix. *Obstetrics and Gynecology*. 1996; 88:927–32. [PubMed: 8942829]
12. Erez N, Truitt M, Olson P, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an nf- κ b-dependent manner. *Cancer Cell*. 2010; 17:135–47. [PubMed: 20138012]
13. Irigaray P, Newby JA, Clapp R, Hardell L, Howard V, Montagnier L, et al. Lifestyle-related factors and environmental agents causing cancer: An overview. *Biomedicine & Pharmacotherapy*. 2007; 61:640–58.
14. Anand P, Kunnumakara A, Sundaram C, Harikumar K, Tharakan S, Lai O, et al. Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research*. 2008; 25:2097–116. [PubMed: 18626751]
15. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, et al. Environmental and heritable factors in the causation of cancer — analyses of cohorts of twins from sweden, denmark, and finland. *New England Journal of Medicine*. 2000; 343:78–85. [PubMed: 10891514]
16. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell*. 2005; 7:211–7. [PubMed: 15766659]

17. Harris RE. Cyclooxygenase-2 (cox-2) and the inflammogenesis of cancer. *Subcellular Biochemistry*. 2007; 42:93–126. [PubMed: 17612047]
18. Liu J, Xu Z, Song Q, Konger RL, Kim YL. Enhancement factor in low-coherence enhanced backscattering and its applications for characterizing experimental skin carcinogenesis. *Journal of Biomedical Optics*. 2010; 15:037011. [PubMed: 20615040]
19. Sahu RP, Dasilva SC, Rashid B, Martel KC, Jernigan D, Mehta SR, et al. Mice lacking epidermal ppargamma exhibit a marked augmentation in photocarcinogenesis associated with increased uvb-induced apoptosis, inflammation and barrier dysfunction. *International Journal of Cancer*. 2012; 131:E1055–66.
20. Wilgus TA, Koki AT, Zweifel BS, Kusewitt DF, Rubal PA, Oberyzy TM. Inhibition of cutaneous ultraviolet light b-mediated inflammation and tumor formation with topical celecoxib treatment. *Molecular Carcinogenesis*. 2003; 38:49–58. [PubMed: 14502644]
21. Pentland AP, Schoggins JW, Scott GA, Khan KN, Han R. Reduction of uv-induced skin tumors in hairless mice by selective cox-2 inhibition. *Carcinogenesis*. 1999; 20:1939–44. [PubMed: 10506108]
22. Xu Z, Liu J, Hong DH, Nguyen VQ, Kim MR, Mohammed SI, et al. Back-directional gated spectroscopic imaging for diffuse light suppression in high anisotropic media and its preclinical applications for microvascular imaging. *IEEE Journal of Selected Topics in Quantum Electronics*. 2010; 16:815–23.
23. Xu Z, Liu J, Kim YL. Diffuse light suppression of back-directional gating imaging in high anisotropic media. *Journal of Biomedical Optics*. 2009; 14:030510. [PubMed: 19566291]
24. Mecklenburg L, Tobin DJ, Muller-Rover S, Handjiski B, Wendt G, Peters EMJ, et al. Active hair growth (anagen) is associated with angiogenesis. *Journal of Investigative Dermatology*. 2000; 114:909–16. [PubMed: 10771470]
25. Elmets CA, Viner JL, Pentland AP, Cantrell W, Lin HY, Bailey H, et al. Chemoprevention of nonmelanoma skin cancer with celecoxib: A randomized, double-blind, placebo-controlled trial. *Journal of the National Cancer Institute*. 2010; 102:1835–44. [PubMed: 21115882]
26. Benavides F, Oberyzy TM, VanBuskirk AM, Reeve VE, Kusewitt DF. The hairless mouse in skin research. *Journal of Dermatological Science*. 2009; 53:10–8. [PubMed: 18938063]
27. Askew DA, Mickan SM, Soyer HP, Wilkinson D. Effectiveness of 5-fluorouracil treatment for actinic keratosis – a systematic review of randomized controlled trials. *International Journal of Dermatology*. 2009; 48:453–63. [PubMed: 19416373]
28. Braakhuis BJM, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of slaughter's concept of field cancerization. *Cancer Research*. 2003; 63:1727–30. [PubMed: 12702551]
29. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer*. 2003; 3:401–10.
30. Ansari KM, Rundhaug JE, Fischer SM. Multiple signaling pathways are responsible for prostaglandin e-2-induced murine keratinocyte proliferation. *Molecular Cancer Research*. 2008; 6:1003–16. [PubMed: 18567804]
31. Tripp CS, Blomme EAG, Chinn KS, Hardy MM, LaCelle P, Pentland AP. Epidermal cox-2 induction following ultraviolet irradiation: Suggested mechanism for the role of cox-2 inhibition in photoprotection. *Journal of Investigative Dermatology*. 2003; 121:853–61. [PubMed: 14632205]
32. Hatton JL, Parent A, Tober KL, Hoppes T, Wulff BC, Duncan FJ, et al. Depletion of cd4+ cells exacerbates the cutaneous response to acute and chronic uvb exposure. *Journal of Investigative Dermatology*. 2007; 127:1507–15. [PubMed: 17363918]
33. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100:57–70. [PubMed: 10647931]

Figure 1. During chemical carcinogenesis, tumors develop in regions of focal hyperemia
 FVB/n mice were treated with a single dose of DMBA and repeated dosing with PMA and then followed for tumor occurrence and sequential imaging. **A)** Visible tumor multiplicity in mice treated with DMBA/PMA. **B)** Averaged Hgb content within the imaging area (acetone)-treated mice shows a significant increase in Hgb content over time for DMBA/PMA treated mice, but not for vehicle treated mice. For the DMBA/PMA group, the slope estimate of the linear regression for Hgb content over time was significantly greater than zero ($p=0.027$). The slope of the regression line for the vehicle group was not significantly different from zero ($p=0.383$). Moreover, there was a significant difference in the slope estimates between DMBA/PMA-treated and vehicle treated mice ($p=0.007$). **C)** Hyperemia precedes grossly visible tumor formation. Top (10 weeks after the first carcinogen treatment): Left – white-light image (no visible lesion), Middle - Hgb content, and Right - threshold mapping to define areas of high Hgb content. Bottom (5 weeks later): Tumors now visible along with expansion of the hyperemic area. Of nine tumors observed in the imaging area, all occurred in areas of persistent hyperemia.

Figure 2. Focal areas of intense hyperemia precede tumor occurrence and serve to indicate sites at high risk for tumor development

A) Color photographic image of an SKH-1 hairless mouse and the tattoo marks (blue) that were used to orient subsequent imaging studies. These tattoo marks are also visible in the Hgb content image. **B)** Celecoxib treatment suppresses tumor formation following UVB treatments. Tumor multiplicity in UVB-treated mice that were also treated with vehicle (Veh) or celecoxib (Coxib) were calculated as the average number of tumors per mouse. **C)** Hgb content images and the corresponding photographs are shown for a representative mouse at 12, 16, and 20 weeks after initiating UVB treatments. Focal areas of increased hyperemia are noted to appear prior to visible tumor appearance (hashed circles). **D)** After the final imaging study (30 weeks after initiating UVB treatments), the irradiated mice were euthanized and skin free of visible tumors were excised from areas of low and high Hgb content. Histopathologic assessment for the presence of small tumors visible microscopically was then done. Total microscopic tumors were normalized to the mean tissue section length for each mouse. The data show the mean tumor density per linear mm in areas of low and high Hgb content for both Veh and Coxib treated animals. Tumors/section length (mm) were significantly higher in areas of high Hgb content relative to low Hgb content for both Veh treated ($p=0.0097$ for a Mann–Whitney U test) and for Coxib treated animals ($p=0.0436$ for a Mann–Whitney U test).

Figure 3. UVB-induced hyperemic foci contain higher Hgb content than areas of high regional blood flow in non-irradiated animals while celecoxib's chemopreventive activity is associated with a reduction in the area of hyperemic foci

A) Representative Hgb content maps are shown for the dorsal surface of a non-irradiated SKH-1 mouse and a mouse 6 weeks after cessation of UVB treatments. **B)** Beginning two weeks after stopping UVB treatments, threshold maps were obtained at 2 week intervals for areas with Hgb content > 1.6 mg/ml. The average Hgb content within the 1.6 mg/ml threshold mapped sites was determined for each mouse at each time point. The time (12–28 weeks) averaged mean and SD is shown for each treatment condition. The differences between UVB irradiated and sham irradiated groups were statistically significant ($p=0.0032$ for Veh and UVB+Veh and $p=0.0073$ for Coxib and UVB+Coxib). **C)** After threshold mapping, the percent of the imaging area containing Hgb greater than 1.6 mg/ml is shown as a time (12–28 weeks) averaged mean and a SD for all animals (5 or 8 animals per treatment group). Celecoxib treatment results in a statistically significant reduction in the total area of involved skin with Hgb content > 1.6 mg/ml ($p=0.0002$ for a Mann–Whitney U test). Note that all visible tumors seen in the imaging area occurred in areas of preexisting hyperemia. Visible tumor occurrence after normalizing to the percent of the imaging area with Hgb content > 1.6 mg/ml is shown below the plot.

Figure 4. UVB-induced hyperemic foci expand following cessation of carcinogenic UVB treatments

A) Representative microvascular Hgb content maps from a single UVB-treated mouse at biweekly intervals after cessation of UVB treatments reveal the spatial and temporal extent of focal hyperemia. Note that hyperemic foci not only persist, but also expand in size leading up to tumor formation. The hashed circles indicate a hyperemic foci that preceded the appearance of a tumor that became visible in week 26 and 28 (arrows). **B)** After threshold mapping for areas with Hgb content > 1.6 mg/ml, the mean area of hyperemic foci was calculated. The areas of high Hgb content expanded after cessation of UVB treatment in both UVB treated mice ($p=0.005$ for the slope estimate over time in UVB+Veh animals and $p=0.029$ in UVB+Coxib mice). The overall difference of focal hyperemic area between the Veh and Coxib treated groups was statistically significant ($p=0.044$). However, the slope estimates between the two groups were not statistically different. **C)** Expanding areas of focally high Hgb content were observed in the weeks preceding visible tumor appearance. The slope estimates of the linear regression for both groups were significant ($p=0.004$ for UVB+Veh and $p=0$ for UVB+Coxib). The difference in the slopes between the two groups was statistically significant ($p=0.021$).

Figure 5. Focal areas of persistent hyperemia exhibit epidermal hyperplasia, the presence of dermal inflammatory infiltrates and increased microvessel density 20 weeks after stopping UVB irradiations

A–D) Representative low power photomicrographs of H&E stained sections from: **A)** Area of low Hgb content in non-irradiated mouse skin. **B)** Area of low Hgb content in irradiated mouse skin. **C)** Area of high Hgb content from non-irradiated skin. **D)** Area of high Hgb content from irradiated skin. **E)** Representative high power photomicrograph showing H&E stained areas of both low and high Hgb content. Note the hypercellular dermis with increased small vessels containing erythrocytes (arrows) in the area of high Hgb content. **F)** Immunohistochemical labeling for the endothelial marker CD31 and MVD determination was performed. Non-UVB treated mice showed no significant difference between areas of high and low regional blood flow or between Veh or Coxib treatment. After subtracting the baseline MVD from the relative non-irradiated control group, the increase in MVD was plotted as the mean and SD (n=4 mice for Veh and n=3 mice for Coxib treatment). A significant difference was noted between the areas of high and low Hgb content for the UVB +Veh treated mice ($p=0.0209$ for a Mann–Whitney U test).

Figure 6. Neither clinically apparent erythema (sunburn) nor averaged subclinical Hgb content predict the site of future tumor formation or the chemopreventive activity of celecoxib

A) Representative microvascular Hgb content images (top) and photographic images (bottom) are shown demonstrating the intense hyperemia (top) and erythema (bottom) that is still evident 72 hours after the final UVB dose. A Hgb content image and photographic image of the same site 17 days later demonstrate resolution of the acute hyperemia and erythema response. Acute hyperemia did not correlate with the future appearance of tumors. The dashed circles indicate a site of a tumor that appeared 10 weeks after stopping UVB treatments. **B)** Erythema was measured using a narrow-band reflectance spectrophotometer (Mexameter®) in the 5th week of UVB treatments (72 hrs after the previous UVB dose) ($p=0.0515$ for a Kruskal–Wallis test). Results represent the mean $\pm$ SD of the erythema measurements ($n=2$ non-irradiated & 5–6 irradiated mice per group).