Tumorigenesis and Neoplastic Progression

ErbB2 Regulates Inflammation and Proliferation in the Skin after Ultraviolet Irradiation

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Exposure to ultraviolet (UV) irradiation is the major cause of nonmelanoma skin cancer, the most common form of cancer in the United States. UV irradiation has a variety of effects on the skin associated with carcinogenesis, including DNA damage and effects on signal transduction. The alterations in signaling caused by UV regulate inflammation, cell proliferation, and apoptosis. UV also activates the orphan receptor tyrosine kinase and proto-oncogene ErbB2 (HER2/neu). In this study, we demonstrate that the UV-induced activation of ErbB2 regulates the response of the skin to UV. Inhibition or knockdown of ErbB2 before UV irradiation suppressed cell proliferation, cell survival, and inflammation after UV. In addition, ErbB2 was necessary for the UV-induced expression of numerous proinflammatory genes that are regulated by the transcription factors nuclear factor- κ B and Comp1, including interleukin-1 β , prostaglandin-endoperoxidase synthase 2 (Cyclooxygenase-2), and multiple chemokines. These results reveal the influence of ErbB2 on the UV response and suggest a role for ErbB2 in UV-induced pathologies such as skin cancer. (Am J Pathol 2006, 169:1402–1414; DOI: 10.2353/ajpath.2006.060082)

UV irradiation causes pathological changes in the skin such as sunburn, skin cancer, and photoaging. These effects are due to both UV-induced DNA damage and altered cellular signaling.¹ UV activates multiple signaling pathways through nongenetic mechanisms, resulting in profound changes in gene expression. This process resembles the response to growth factors and is known as the UV response. Much of the UV response is due to the activation of mitogen-activated protein kinase (MAPK) family members and NF- κ B (nuclear factor- κ B) pathways. NF- κ B induction of numerous cytokines leads to edema and erythema, two components of UV-induced

inflammation. NF- κ B also regulates cell proliferation and cell death in response to UV. MAPK family members can activate I κ B α kinase, a key step in the activation of NF- κ B (reviewed in Ref. 2). Activation of MAPK cascades culminates in signal transduction to the nucleus and transcription of immediate early genes necessary for cell proliferation.

MAPKs are downstream from many receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) family of receptors. Interestingly, UV exposure activates EGFR family members through a mechanism involving reactive oxygen species. Reactive oxygen species-mediated inactivation of receptor-associated phosphatases is believed to block receptor deactivation.³ UV exposure also alters receptor degradation^{3–6} and increases EGFR ligand expression.^{7,8} Activation of EGFR by UV stimulates both MAPK and phosphatidylinositol 3-kinase/Akt signaling.⁹ EGFR activation also suppresses apoptosis, increases proliferation, delays hyperplasia, and increases skin carcinogenesis following UV exposure.^{4,9–13}

Although most research has focused on the influence of EGFR on the UV response, three members of the EGFR family are expressed in the skin and activated by UV. They include EGFR itself, ErbB2 (HER2/neu), and ErbB3. Overexpression of ErbB2 in the skin results in epidermal and follicular hyperplasia and spontaneous papilloma formation.^{14–16} In addition, the receptor is overexpressed in many human cancers including breast, colon, and squamous cell carcinoma and is associated with a poor prognosis and resistance to chemotherapy in human adenocarcinomas (reviewed in Ref. 17).

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Because of its role in carcinogenesis, we hypothesized that ErbB2 activation by UV may regulate the UV response of the skin. To assess the role of ErbB2 activity in UV-exposed skin, the ErbB2-specific tyrophostin inhibitor AG825 was used to block the UV-induced activation of ErbB2. Transcriptional regulation by ErbB2 in response to UV exposure was examined *in vivo* using microarray analysis. The microarray analysis, together with cell biology experiments, demonstrated that ErbB2 regulates inflammation, proliferation, and, to a lesser extent, apoptosis following UV irradiation and suggests that these effects are modulated by ErbB2 activation of NF- κ B and Comp1 (cooperates with myogenic protein 1) pathways. We propose that repeated cycles of ErbB2 activation on chronic UV exposure deregulates the response of the skin to UV, contributing to UV-induced skin pathologies such as cancer.

Materials and Methods

Animals

Adult female CD-1 mice were maintained in our animal facility and provided with Purina lab chow (Nestlé Purina PetCare, St. Louis, MO) and water *ad libitum*. The dorsal skin was clipped 1 day before treatment and shaved with a Remington Microscreen shaver (Wahl, Sterling, IL) on the day of treatment. Dimethyl sulfoxide (DMSO) or 4 mg of AG825 (AG Scientific, San Diego, CA) dissolved in DMSO was applied topically to the shaved backs of the mice 2 hours before exposure to 10 kJ/m² UV or sham irradiation. UVB TL 40W/12 RS bulbs (Philips, Somerset, NJ) were used that emitted approximately 30% UV-A, 70% UV-B, and <1% UV-C, with a total output of 470 μ W/cm², as measured with radiometric photodetector probes (Newport, Irvine, CA). Skin-fold thickness was measured using calipers to lightly pinch the dorsal skin. All animal procedures were approved by our Institutional Animal Care and Use Committee.

Cell Culture

Primary keratinocytes were isolated from newborn CD-1 mouse skin as described previously.¹⁸ In brief, the skin was floated on trypsin (Invitrogen, Carlsbad, CA) at 4°C overnight; the epidermis separated from the dermis; and the epidermis minced, triturated, and centrifuged in SMEM (Invitrogen) containing 8% fetal bovine serum (HiCa media) and penicillin/streptomycin (Invitrogen). The cells were plated in the same medium and were refed the next day and every 2 to 3 days thereafter with SMEM containing 10% chelexed fetal bovine serum with a calcium concentration adjusted to 0.05 mmol/L and penicillin/streptomycin. Keratinocytes grown to 70% confluence were exposed to 600 J/m² UV or sham-irradiated in a thin layer of phosphate-buffered saline containing 0.05 mmol/L calcium. Some keratinocytes were pretreated with 45 μ mol/L AG825 dissolved in DMSO or DMSO alone 2 hours before UV

exposure and refed fresh medium containing AG825 or DMSO immediately after UV exposure. Transfection with ErbB2-targeted small interfering RNA (siRNA) (5'-GCAACACCCAUCUCUGCUUUGUAAA-3'), Stealth RNAi Negative Control LO GC (Invitrogen), Cy3-conjugated Label IT RNAi Delivery Control (Mirus, Madison, WI), and sham transfection was performed with TransIT-siQUEST Transfection Reagent (Mirus) according to the manufacturer's protocol. Transfection efficiency was quantified by determining the proportion of the cells that incorporated the fluorescent Cy3-conjugated siRNA 1 day after transfection.

Microarray Analysis

Flash frozen skin was ground to a powder using a mortar and pestle and RNA extracted using a Power-Gen 700 tissue homogenizer (Fisher, Hampton, NH) in TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was further purified using RNeasy Midi Columns according to the manufacturer's protocol (Qiagen, Valencia, CA). The quality of the RNA was assessed using an RNA 6000 Pico Assay (Agilent, Palo Alto, CA) with a Bioanalyzer (Agilent). Mouse MOE430A gene chips were purchased from Affymetrix (Santa Clara, CA). Five micrograms of total RNA from each sample was reverse-transcribed using Superscript II (Invitrogen). *In vitro* transcription to generate biotinylated cRNA was performed using the Bioarray High Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY). Fifteen micrograms of fragmented cRNA was hybridized for 16 hours to mouse MOE430A chips at 45°C using the Affymetrix 640 hybridization oven, stained, and scanned using the Agilent scanner according to standard Affymetrix protocols. Signal intensities from the Affymetrix ".CEL" files were derived using GeneChip Operating Software v1.2 (Affymetrix), multiplicative model-based expression index (dChip software),¹⁹ and robust multi-array average.²⁰ The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE4066. Methods used to determine significant changes in gene expression from the derived signal intensities were rank products,²¹ significance analysis of microarrays,²² analysis of variance (analysis of variance), Student's *t*-test, and fold-change. A list of genes whose expression was altered using each method was created and only genes appearing on all lists were considered significantly altered and reported (Supplemental Table 1, see <http://ajp.amjpathol.org>). Hierarchical clustering of gene expression was performed with dChip software. Samples not exposed to UV were significantly clustered together ($P \leq 0.05$) versus all other samples suggesting similarities in gene expression among those samples. Significant clustering was also found among samples 6 hours after UV and among samples 24 hours after UV. Furthermore, significant

clustering was found among samples treated with DMSO and among those treated with AG825. Scatter plots were generated using GeneChip Operating Software. Self-organizing maps were generated using GeneCluster 2.0 software.²³ Grouping of genes into biological processes was performed using NetAffx Analysis Center (<http://www.affymetrix.com>) and literature searches. Genes were mapped into biological networks with Pathways Analysis software (Ingenuity Systems, Mountain View, CA). Promoter Analysis and Interaction Network Toolset v3.3 (PAINT) was used to scan sequences up to 2000 bp upstream of genes with altered expression to search for transcription factor binding sequences or transcriptional response elements (TRE) using a 0.95 core similarity threshold.²⁴ The clustering option through PAINT was used to visualize over-represented Gene-TRE networks using only significantly over-represented TRE ($P \leq 0.05$).

Real-Time PCR

Selected changes in gene expression were validated using real-time PCR. Sequences supplied by Affymetrix from the probe set of *Il1b* (probe: 1449399_a_at), *Mmp9* (probe: 1416298_at), and *Thbs1* (probe: 1421811_at) were scanned for unique sequences using a stringent BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences spanning exons were selected preferentially for probes and primers for prepared using Assays-by-Design (Applied Biosystems, Foster City, CA). Real-time PCR was performed using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) according to the manufacturer's instructions on an ABI Prism 7000 (Applied Biosystems). The relative efficiency of amplification of the selected gene versus control (GAPDH or actin) was plotted to ensure the slope of total RNA versus ΔC_T was less than 0.05. The $2^{-\Delta\Delta C_T}$ was used to determine fold-change differences between samples.²⁵

Immunoblotting

Flash-frozen skin was ground with a mortar and pestle on dry ice and homogenized in lysis buffer containing 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L EDTA, Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany), 1 mmol/L Na_3VO_4 , 1.5 $\mu\text{mol/L}$ EGTA, and 10 $\mu\text{mol/L}$ NaF. For some experiments, the epidermis was separated from the skin by the heat shock method²⁶ before homogenization. Keratinocytes were lysed in the same buffer. Protein was quantified using the Coomassie Brilliant Blue G-250 protein assay (Bio-Rad, Hercules, CA). The evenness of loading and transfer was determined by staining with Ponceau S and by actin immunoblotting. Membranes were immunoblotted with antibodies recognizing Erbb2, phospho-Erbb2 (Tyr¹²⁴⁸), EGFR, phospho-EGFR (Tyr⁹⁹²), phospho-EGFR (Tyr¹¹⁷³), phosphotyrosine, NF- κB (Cell Signaling, Beverly, MA), actin (Sigma, St.

Louis, MO), and Tal1 (Calbiochem, San Diego, CA). Binding of horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) was visualized using chemiluminescent reagents (Pierce, Rockford, IL) and autoradiography. Densitometry was performed using 1DScan software (Scanalytics, Fairfax, VA).

Proliferation and Apoptosis Assays

Proliferation, survival, and apoptosis were quantified in three replicate experiments. 5-Bromo-2'-deoxyuridine (BrdU) uptake was measured by a chemiluminescent BrdU cell proliferation enzyme-linked immunosorbent assay (Roche). Survival was quantified using a 4-methylumbelliferyl heptanoate (MUH; Sigma) degradation product fluorescence (ex: 360 nm, em: 465 nm) as a marker for cell viability based on the method by Dotsika et al.²⁷ Cells were incubated in 100 $\mu\text{g/ml}$ MUH in phosphate-buffered saline for 30 minutes at 37°C. MUH fluorescence from wells lacking cells was subtracted from all samples. Apoptosis was measured using an ssDNA apoptosis enzyme-linked immunosorbent assay kit (Chemicon, Temecula, CA).

Results

AG825 Inhibits the UV-Induced Activation of Erbb2

Exposure of mouse skin to UV rapidly activated Erbb2, as measured by the phosphorylation of Erbb2 on Tyr¹²⁴⁸. Erbb2 phosphorylation in the epidermis was maximally increased more than twofold by 5 minutes after UV, with a second smaller peak of activation at 30 minutes (Figure 1, A and B). By 40 minutes, Erbb2 phosphorylation had returned to constitutive levels (Figure 1A). The UV-induced activation was due, in part, to an increase in the total level of Erbb2 (Figure 1B). To determine the effect of Erbb2 activation on transcription following UV, a method for the pharmacological inhibition of Erbb2 *in vivo* was developed. Topical treatment of mice with the tyrphostin Erbb2 inhibitor AG825 prevented the UV-induced phosphorylation of Erbb2 at 30 minutes after UV (Figure 1B). The decrease in Erbb2 activity was disproportionately greater than the observed decrease in total receptor content (Figure 1B). By 24 hours after UV, phosphorylated Erbb2 in AG825-treated epidermis was still somewhat reduced compared with the corresponding vehicle-treated control, and the total Erbb2 protein was decreased (Figure 1B).

Because Erbb2 transduces signals through the formation of heterodimers with other family members, the effect of inhibition of Erbb2 on EGFR and Erbb3 activation after UV exposure was determined. AG825 pretreatment blocked the UV-induced phosphorylation of EGFR on Tyr⁹⁹² but not Tyr¹¹⁷³ (Figure 1B). Inhibition of Erbb2 also prevented the increased phosphorylation of Erbb3 on Tyr¹²⁸⁹ that occurred 30 minutes after UV exposure. AG825 also blocked the phosphorylation of proteins at

Figure 1. UV exposure results in the rapid activation of Erb2 and is reduced by the inhibitor AG825. **A:** Mice were exposed to 10 kJ/m² UV and epidermal protein extracts, obtained at the indicated times after UV, were immunoblotted as indicated (top panel), and relative Erb2 phosphorylation was quantified by densitometry (bottom panel). Experiment shown in top panel is representative of three replicate experiments performed and quantified (bottom). **B:** Mice were topically treated with DMSO alone or AG825 in DMSO, exposed to 10 kJ/m² UV, and euthanized at the indicated time points. **A** and **B:** Immunoblotting was performed with the indicated antibodies. Expression relative to actin as quantified by densitometry is shown. **Arrow** indicates the location of the EGFR family (including Erb2), and graph indicates results of densitometry performed on the seven strongest bands detected, not including Erb2. **P* ≤ 0.05, significantly different versus time 0 using a Student's *t*-test.

~180 kD, consistent with the molecular weight of the EGFR family, but did not alter tyrosine phosphorylation of proteins at other molecular weights (Figure 1B).

Figure 2. Experimental design. Groups of mice were treated as shown and as described in Materials and Methods. At the indicated times, mice were euthanized and the skin removed for analysis. *N* = 4 mice per group.

Erb2 Regulates the Transcription of Many Genes following UV Irradiation

The influence of Erb2 on gene expression following UV exposure was assessed using microarray analysis. As described in Figure 2, skin from mice treated with the Erb2 inhibitor AG825 or the vehicle alone before each of two UV or sham exposures was harvested either 6 or 24 hours after UV. Mice were exposed to UV twice because previous results indicated the influence of Erb2 receptors on the response of the skin to UV increases with multiple exposures.⁹ A measure of the interanimal variation among the mice in each group is revealed by scatter plots comparing gene expression in two mice from the same group. In two randomly selected vehicle-treated and sham-irradiated mice, eight genes of approximately 13,000 detected (<0.1%) were expressed with more than a fivefold difference between them (Figure 3A). Similar results were observed with UV-exposed or AG825-treated skin (data not shown). In contrast to the comparison of gene expression between samples of the same group, a plot of AG825-treated skin compared with vehicle-treated skin produced a much wider scatter (Figure 3B). Many of the data points are shifted upwards, indicating that inhibition of Erb2 more often increased rather than decreased gene expression. This trend occurred in both sham- and UV-irradiated skin (Figure 3, B and C). Regardless of inhibitor treatment, the scatter plot of UV-exposed compared with sham-irradiated skin was much broader than any other plot (Figure 3D).

Further analysis of the microarray data produced a list of 361 genes whose expressions changed significantly on abrogation of Erb2 activity (Supplemental Table 1, see <http://ajp.amjpathol.org>). The expression of 61% of these genes was increased on Erb2 inhibition. Inhibition of Erb2 without UV irradiation altered the expression of 39 genes, with 64% of these increased on AG825 treat-

Figure 3. Scatter plots reveal patterns of gene expression. Gene expression intensity from one mouse was plotted against the gene expression intensity of another mouse for all genes probed, both on a log scale. Each red dot is a gene that was expressed ("present") in both mice. Blue dots are considered present in one mouse and absent in the other. Yellow dots are genes considered absent in both mice. Lines represent 2-, 5-, 10-, and 25-fold change. The width of the plot along the $y = x$ line demonstrates a rough measure of variability in gene expression between the two mice with a narrow scatter indicating genes were expressed at similar intensities. Mice indicated as being exposed to UV were from the 6-hour group.

ment. Inhibition of *ErbB2* before UV exposure altered the expression of 218 (61% with increased expression) and 127 genes (54% with increased expression) at 6 and 24 hours, respectively. Thus, *ErbB2* activation more frequently decreased rather than increased gene expression after UV.

ErbB2 Regulates the Expression of Genes Important in Many Biological Processes

To determine the biological significance of *ErbB2* activation in UV-exposed skin, gene ontology mining, and other search strategies were performed to associate changes in gene expression with biological processes. Statistical analysis was performed on the probability of the number of genes occurring in these processes (Supplemental Table 2, see <http://ajp.amjpathol.org>). Changes in the expression of 158 genes with a function in metabolism accounted for the largest percentage (44%) of the changes. Many genes important in the immune response (65 genes), cell communication (65 genes), cancer (55 genes), adhesion and migration (47 genes), development (55 genes), proliferation (36 genes), and apoptosis (25 genes) were also regulated by AG825 (Table 1). Six genes relating to pigmentation, mainly melanin biosynthesis, were revealed by the microarray analysis, comprising one-third (6 of 18) of the genes in the Mouse Genome Informatics database known to be involved in pigmentation.

Changes in gene expression over time were clustered and trends in the data graphed in self-organizing maps, revealing distinct patterns of gene expression on inhibition of *ErbB2*. Several prominent patterns were evident, including delayed up- or down-regulation (Figure 4, A and B) or a more sustained decrease (Figure 4C) or increase (Figure 4D) in gene expression after UV in the inhibitor-treated skin compared with the corresponding control. The expression of many cell-cycle regulatory genes such as *Ccna2*, *Ccnb1*, and *Ccnb2* (*cyclin A2*, *B1*, and *B2*) followed the pattern in Figure 4H, demonstrating a lesser responsiveness to UV after AG825 treatment. Genes associated with inflammation such as *Il1b* (*Interleukin-1β*), *Ptgs2/COX2* (*Prostaglandin-endoperoxidase synthase 2/Cyclooxygenase-2*), *Sell* (*lymphocyte adhesion modulator L-selectin*), *Ccl4*, *Cxcl2*, and *Cd44* all fit the pattern in Figure 4I, demonstrating an *ErbB2*-dependent induction after UV. The expression of genes with altered expression in tumorigenesis such as *Hif1a* (*hypoxia inducible factor 1, α subunit*), *Spp1* (*secreted phosphoprotein 1*),

Table 1. Inhibition of *ErbB2* and UV Exposure Alters the Expression of Genes Involved in Various Biological Processes

Biological process	Sham		6 hours after UV		24 hours after UV		Total*
	↓	↑	↓	↑	↓	↑	
Proliferation	3 [†]	2	6	21	3	6	36 (10%) [‡]
Apoptosis	2	2	6	5	4	6	25 (7%)
Immune response	0	8	42	3	14	4	65 (18%)
Adhesion and migration	0	7	13	14	14	4	47 (13%)
Cancer	3	5	12	25	7	8	55 (15%)
Metabolism	10	9	23	63	24	43	158 (44%)
Development	2	6	8	26	12	4	55 (15%)
Pigment biosynthesis	3	0	0	5	0	1	6 (2%)
Cell communication	1	6	18	18	20	5	65 (18%)
Total genes	14	25	85	133	59	68	361

*Note that because the expression of some genes is changed at multiple times, the total may not reflect the sum of the individual time points.

[†]Number of genes increased (↑) or decreased (↓) in AG825-treated samples compared with the vehicle alone.

[‡]Numbers in parentheses indicate the percentage of the total number of genes altered by *ErbB2* for each biological process.

Figure 4. Self-organizing maps reveal trends in gene expression after UV exposure. Each graph represents a cluster of genes with similar expression patterns. The $\log_2$ gene expression intensity mean for each treatment group was graphed over time. The first three points represent the DMSO-treated group. The next three points represent the AG825-treated group. Within each cluster, the black dots and connecting lines represent the centroid (average pattern) of gene expression, whereas the bracketing lines indicate the SD. Sh, sham irradiation, and the numbers indicate the time (h) after UV. *The point at which there is significant difference in gene expression on inhibition of ErbB2.

and *Ghr* (growth hormone receptor) followed this pattern as well. The microarray data revealed that 54 genes implicated in cancer were modulated by ErbB2, most with reduced expression after ErbB2 inhibition. The vast majority of genes were not significantly altered by inhibitor treatment or UV exposure (Figure 4J).

Although genes involved in many biological processes generally had expression profiles dispersed among the patterns shown in Figure 4, A–I, two consistent trends appeared. The most predominant of these was the large number of inflammation-associated genes whose UV-induced expression was blocked by the ErbB2 inhibitor (Figure 4I). The second most prevalent pattern was a lesser decrease of proliferation- and apoptosis-associated genes after UV in samples treated with the ErbB2 inhibitor (Figure 4H). These data suggest ErbB2 modulates gene expression in the skin after UV, favoring inflammation and playing a role in proliferation and apoptosis.

ErbB2-Regulated Genes Have Binding Sites for Several Transcription Factors

PAINT analysis of the microarray data was used to identify the transcription factors responsible for ErbB2's effects on gene expression. PAINT analyzed the TRE occurrences for over- or under-representation in our gene lists compared with the frequency predicted by examining all genes present on the microarray. Four TREs were identified as being significantly over-represented on a subset of 29 genes identified by microarray analysis (Figure 5A). Clustering of the data linked ErbB2-regulated genes with these particular transcription factors and indicated their relatedness. *Tal1* (*T-cell acute lymphocytic leukemia 1*) ($P < 0.05$), which was found

Figure 5. PAINT analysis reveals novel transcription factors regulated by ErbB2. **A:** Two-way clustering of TREs versus genes decreased by ErbB2 inhibition was performed via PAINT analysis. Significantly overexpressed TREs versus genes in which they were found upstream are shown. Significance of $P \leq 0.05$ was determined as in Ref. 24. **B** and **C:** Mice were treated with DMSO or AG825, exposed to 10 kJ/m² UV, and euthanized 6 hours later. Whole-skin protein extracts were obtained, immunoblotted for the indicated antibody, and quantified by densitometry relative to actin expression. $N = 4$ mice. *Gene symbols listed here and not defined in the text conform to the Mouse Genome Informatics 3.41 database nomenclature. ** $P \leq 0.05$, significantly different using a Student's *t*-test.

Figure 6. Real-time PCR validates the microarray results. Gene expression changes in three highly expressed genes, as indicated by microarray analysis, were validated using real-time PCR. Two-way analysis of variance analysis indicated significant changes of the same magnitude using microarray expression analysis or real-time PCR of *Il1b*, *Mmp9*, and *Thbs1* and no differences between the methods of analysis (microarray versus real-time PCR). $N = 3$ mice. Data are expressed as fold change compared with sham-irradiated and DMSO-treated skin. * $P \leq 0.05$, significantly different using a two-way analysis of variance.

upstream of the cancer-associated genes *Pla1a* (phospholipase A1 member A) and *Tde1* (tumor differentially expressed 1), was the most closely related transcription factor to NF- κ B (Figure 5A). Consistent with this result, immunoblotting revealed that Tal1 protein was decreased by 70% on inhibition of Erbb2 in sham-irradiated mouse skin (Figure 5B). A binding site for the transcription factor NF- κ B ($P = 0.01$), known for its role in inflammation, proliferation and apoptosis (reviewed in^{2,28}), was significantly over-represented upstream of seven genes whose expression was regulated by Erbb2 (Figure 5A). Several of these genes have a role in inflammation, including *Il1b*, *Tde1*, *Ptgs2*, *H2-T23* (histocompatibility 2, T region locus 23), *Cxcl2*, and *Cxcl10* (Figure 5A). Consistent with the PAINT analysis, immunoblotting of sham-irradiated mouse skin revealed a 65% decrease in NF- κ B protein on inhibition of Erbb2 (Figure 5C). A Comp1 binding site was significantly over-represented ($P = 0.01$) upstream of 16 genes, nine of which were related to inflammation. These included *Hmr* (Homerin), *ligp1* (Interferon inducible GTPase 1), *Tra1* (Tumor rejection antigen gp96), *Tgtp* (T-cell-specific GTPase), *Ccl4*, *Pfc* (Properdin factor, complement), *Mgl1* (macrophage galactose N-acetyl-galactosamine specific lectin 1), *Lcp2* (lymphocyte cytosolic protein 2), and *H2-Eb1* (histocompatibility 2, class II antigen Eb). These data suggest a role for Comp1, a little-investigated transcription factor, in the Erbb2-regulated, UV-induced inflammatory response. *FoxJ2* (Forkhead box J2, $P = 0.02$) was the least closely related and found upstream of Erbb2-regulated genes involved in diverse processes (Figure 5A). In summary, our analysis indicated that the transcription factors NF- κ B, Tal1, and Comp1 may lead to increased expression of proinflammatory genes on UV exposure.

Real-Time PCR Validates the Microarray Analysis

The microarray results were validated using real-time PCR of selected genes whose expression was altered at least twofold after AG825 treatment, consistent with the pattern in Figure 4I. *Il1b* expression, similar in sham-irradiated AG825- and vehicle-treated skin, was increased to a greater extent after UV exposure in the vehicle-treated compared with AG825-treated samples in both the real-time PCR (sixfold greater increase) and the microarray experiments (16-fold greater increase) (Figure 6A). Analysis of the Affymetrix data for *Mmp9* (Matrix metalloproteinase 9) detected 3.8- and twofold increases in *Mmp9* expression 24 hours after UV exposure of vehicle- and inhibitor-treated skin, respectively (Figure 6B). Consistent with these results, quantitative PCR found 3.2- and 1.3-fold increases in *Mmp9* expression in the vehicle- and AG825-treated mice 24 hours after UV exposure, respectively (Figure 6B). Both the Affymetrix probe and real-time PCR for *Thbs1* (Thrombospondin 1) detected a significant twofold higher *Thbs1* expression in AG825-treated and sham-irradiated skin compared with the corresponding control but similar *Thbs1* expression in vehicle- and inhibitor-treated mice at 24 hours after UV (Figure 6C). In addition, real-time PCR of *Padi3* (peptidyl arginine deiminase type III), *Chi3l3* (chitinase 3-like 3), and *Socs3* (Suppressor of cytokine signaling 3) yielded statistically similar results compared with the microarray data (data not shown). These data validate the microarray analysis for identification of altered gene expression.

Inhibition of *ErbB2* Suppresses UV-Induced Inflammation

Of biological processes important in the response to UV, the immune response category had the second most number of genes whose expression was modulated by *ErbB2*. Of particular interest was the large number of genes whose expression was lower in *ErbB2* inhibitor-treated skin 6 hours after UV exposure (42 genes) compared with only three genes with higher expression (Table 1). These results deviated sharply from the general trend of increased gene expression on inhibition of *ErbB2*. Cytokines, chemokines, and inflammatory cell markers, many of which are regulated by NF- κ B and Comp1, were included among these genes. *Il1b* and *Ptgs2* are examples inflammatory mediators whose expression was blocked by inhibition of *ErbB2* (Figure 4I). The UV-induced increase in *Il1b* mRNA previously documented and associated with NF- κ B activity (reviewed in Ref. 29) was largely dependent on *ErbB2* in both microarray and real-time PCR experiments (Supplemental Table 1, see <http://ajp.amjpathol.org> and Figure 6A). *Ptgs2*, downstream from NF κ B (Figure 5A), was increased in keratinocytes after UV (reviewed in Ref. 29). Inhibition of *ErbB2* suppressed or delayed the UV-induced expression of other inflammatory cell markers such as the mast cell markers *Cd48* and *Cd53* (Figure 4A), *Sell* (Figure 4I), the chemoattractant *Ptpcr* (protein tyrosine phosphatase, receptor type, C) (Figure 4A), and chemokines such as *Ccl4* (Figure 4I), *Ccl11* (Figure 4A), and *Ccl12* (Figure 4G) (also known as MIP-1 β , eotaxin, and MCP-5, respectively) 6 hours after UV exposure (Supplemental Table 1, see <http://ajp.amjpathol.org>). By 24 hours after UV exposure, the expression of proinflammatory chemokines such as *Cxcl2* (Figure 4I) and *Cd44* (Figure 4I), involved in inflammatory skin disorders, was lower in AG825-treated skin compared with vehicle-treated skin. This analysis predicts an important role for *ErbB2* in the potentiation of the immune response following UV exposure.

To further investigate the role of *ErbB2* in inflammation following UV exposure, skin-fold thickness as a measure of edema was quantified in mice treated with DMSO or AG825 and exposed to UV. As shown in Figure 7, UV exposure maximally increased skin-fold thickness 4 to 5 days after UV. AG825 suppressed UV-induced edema by 33, 37, and 64% at 4, 5, and 6 days, respectively, after UV exposure (Figure 7). Collectively, these results indicate that activation of *ErbB2* by UV augments UV-induced inflammation through NF- κ B- and Comp1-regulated gene expression.

Inhibition of *ErbB2* Decreases Proliferation after UV Exposure

The microarray analysis revealed that *ErbB2* regulates the expression of many genes involved in proliferation. The altered expression of cell proliferation-associated genes occurred primarily at 6 hours after UV (Table 1). At this time point, inhibition of *ErbB2* increased the expression of 21 proliferation-associated genes and decreased

Figure 7. Inhibition of *ErbB2* suppresses UV-induced inflammation. Shaved CD-1 mice were treated with AG825 or vehicle alone and exposed to 10 kJ/m² UV at 0 days. Skin-fold thickness at the treatment area was measured daily. *N* ≥ 8 mice. **P* ≤ 0.01, ***P* ≤ 0.001, significantly different using a two-way analysis of variance.

the expression of only six genes compared with the corresponding vehicle-treated control. Cyclins (cyclins A2, B1, B2, D1, and D2), *Cdc2a* (Cell division cycle 2 homolog), *Cdk4* (Cyclin-dependent kinase 4), and the proliferation marker *Tk1* (thymidine kinase 1) displayed delayed or lesser decreases in expression after AG825 treatment at 6 hours but also less recovery at 24 hours (Figure 4, B and H, respectively). These patterns of expression suggested that proliferation would decrease sharply at 6 hours and begin to recover by 24 hours after UV in vehicle-treated mice, whereas the inhibitor-treated mice would have less of a decrease at 6 hours but potentially no recovery in proliferation at 24 hours.

The role of *ErbB2* in proliferation following UV exposure was investigated in cultured primary keratinocytes using pharmacological or genetic means to block *ErbB2* activity. AG825 treatment blocked the UV-induced phosphorylation of *ErbB2* (Figure 8A). *ErbB2* receptor expression and activity was ablated by transfection of primary keratinocytes with *ErbB2*-targeted siRNA (Figure 8B). BrdU incorporation was assessed in inhibitor-treated or siRNA-transfected keratinocytes after UV exposure and normalized to viable cells using an MUH viability assay. As shown in Figure 8C, BrdU incorporation was significantly decreased in sham-irradiated and AG825- or *ErbB2* siRNA-treated keratinocytes compared with the corresponding controls. UV exposure reduced BrdU incorporation in both DMSO- and AG825-treated keratinocytes by 60 and 70%, respectively, by 6 hours (Figure 8C). However, BrdU incorporation rebounded after 6 hours in control but not AG825-treated or *ErbB2* siRNA-transfected keratinocytes (Figure 8C). These cell culture data validated the predictions formulated by microarray analysis and indicate a role for *ErbB2* in proliferation following UV exposure.

ErbB2 Suppresses Apoptosis after UV Exposure

Although EGFR has been shown to decrease apoptosis in the skin after UV,⁹ it was expected that *ErbB2* may also play a role in modulating apoptosis in the skin after

Figure 8. Inhibition of ErbB2 decreases proliferation and increases apoptosis after UV exposure. **A:** DMSO- or AG825-treated keratinocytes were exposed to 600 J/m² UV and harvested at the indicated time points. **B:** Keratinocytes were transfected with ErbB2-specific siRNA (+) or a negative control siRNA (-). Averages of densitometry results from two or more experiments are shown. **A and B:** Immunoblotting was performed with the indicated antibodies. Expression relative to actin as quantified by densitometry is shown. **C:** Keratinocytes were treated with DMSO, AG825, ErbB2 siRNA, or a negative control siRNA and exposed to UV. BrdU incorporation was measured via anti-BrdU-POD luminescence. In replicate wells, survival was measured via MUH fluorescence at 405 nm. **D:** Keratinocytes were treated with DMSO, AG825, ErbB2 siRNA, or a negative control siRNA and exposed to UV. After UV exposure, the DNA was denatured and incubated with a monoclonal antibody to ssDNA. Absorbance at 360 nm of a peroxidase-conjugated secondary is shown. *N* = 40 wells. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, significantly different from the DMSO- or negative control siRNA-treated controls using a two-way analysis of variance.

UV. Microarray analysis demonstrated that ErbB2 modulates the expression of 25 apoptosis-associated genes, most of them after UV exposure (Table 1). Genes that were decreased to a lesser extent after AG825 (Figure 4H) included proapoptotic genes such *Pawr* (*PRKC*, *apoptosis*, *WT1 regulator*) and *Sox4* (*SRY-box containing gene 4*), as well as markers of apoptosis such as *Pdcd4* (*programed cell death 4*) (Supplemental Table 1, see <http://ajp.amjpathol.org>). The antiapoptotic genes *Ghr* and *Ptgs2* were suppressed by the ErbB2 inhibitor (Supplemental Table 1, see <http://ajp.amjpathol.org>). These changes in gene expression predicted increased apoptosis after UV in the absence of ErbB2 signaling.

To investigate the role of ErbB2 in apoptosis following UV exposure, keratinocyte survival was measured in AG825-treated or ErbB2 siRNA-transfected cells and controls. As expected, apoptosis increased in all groups

after UV exposure, as detected by ssDNA quantification (Figure 8D). AG825- and DMSO-treated keratinocytes exhibited similarly increased ssDNA between 6 and 12 hours after UV (Figure 8D). However, by 24 hours after UV-exposure, apoptosis was significantly increased in the AG825-treated compared with DMSO-treated cells (Figure 8D). Similar results were obtained in keratinocytes transfected with ErbB2-targeted siRNA (Figure 8D). Taken together, these results demonstrate that ErbB2 suppresses UV-induced apoptosis.

Discussion

Previous studies designed to assess the global effects of UV on the skin have used microarray analysis performed on primary keratinocytes and epidermis exposed to

UV.^{30–35} Consistent with our results, these studies have determined that UV modulates hundreds of genes involved in diverse processes such as proliferation, apoptosis, inflammation, cell adhesion, and migration. Using global expression analysis by Affymetrix microarray, we have characterized the transcriptome in the skin modulated by the transient activation of Erb2 after UV exposure and identified many novel genes regulated by Erb2. In addition, our analysis revealed that Erb2 modulates proliferation and inflammation with a lesser but statistically significant effect on apoptosis following UV irradiation.

Stringent criteria were used in this analysis. The inherent variability among *in vivo* samples led to the statistical determination of the need for microarray experiments to be performed at least in triplicate.³⁶ We used biological replicates in quadruplicate for an additional margin of confidence. Microarray analysis is complex, and no two articles seem to use identical criteria. Therefore, multiple sets of criteria were used both to determine probe intensities and to identify genes that were significantly changed. Only genes that met all these criteria were reported herein. Real-time PCR validated the microarray data, whereas scatter plots and clustering revealed global trends in gene expression. Selected biological pathways were validated experimentally, which further confirmed the *in silico* analysis.

Because receptor tyrosine kinases like Erb2 are generally thought to activate signal transduction cascades leading to transcription factor activation, gene expression was expected, for the most part, to be decreased on Erb2 inhibition. Contrary to what was expected, gene expression was more often increased than decreased after its inhibition. Thus, UV-induced activation of Erb2 did more to suppress or inhibit global gene expression than to increase transcription. There were notable exceptions to this trend, however, such as the suppression of expression of numerous immune response genes on inhibition of Erb2 and UV exposure. The significance and mechanisms of suppression of gene expression by Erb2 remain to be determined. Interestingly, microarray analysis of *Egfr*-null and wild-type control skin revealed a similar pattern of suppression of gene expression by the receptor (J.G. Madson, C. Sorensen, and L.A. Hansen, unpublished data). Published studies comparing gene expression in cells with normal and increased Erb2 expression have found varied responses in global gene expression. Overexpression of Erb2 led to either more frequently increased³⁷ or decreased³⁸ gene expression in mammary luminal epithelial cells. Gene expression was more frequently increased in Erb2-overexpressing breast cancer cell lines, but in primary breast tumors, gene expression was more frequently decreased when Erb2 is overexpressed.⁴¹ Therefore, no consensus about the global effects of Erb2 on gene expression has yet emerged from these studies.

A novel role for Erb2 in the induction of inflammation after UV was identified. We found that transient Erb2 activation augments UV-induced inflammation and regulates the expression of a myriad of effectors of this response. Many of these effectors are known to be regu-

lated by NF- κ B, consistent with the PAINT analysis indicating NF- κ B as an Erb2-regulated transcription factor. Two NF- κ B-regulated candidates (reviewed in Ref. 42) for Erb2-induced inflammation after UV exposure are *Il1b* and *Ptgs2*, both potent mediators of inflammation. The UV-induced expression of both *Il1b* and *Ptgs2* was largely dependent on Erb2 activation. *Ptgs2* has also been shown to be regulated by Erb2 in colorectal cells.⁴³ A potential mechanism for Erb2 activation of NF- κ B is the phosphatidyl inositol 3-kinase pathway, as documented in mammary epithelial cells (reviewed in Ref. 28). Erb2 may also suppress the inhibition of NF- κ B by *Pawr*.⁴⁴ Our data also link Erb2's proinflammatory effects to the little-investigated Comp1, because 7 Erb2-regulated, proinflammatory genes had Comp1 binding sites. These genes included *Ccl4*, which is important in acute inflammatory responses and also plays a role in wound reepithelialization and collagen synthesis.⁴⁵ The mechanisms through which Erb2 alters NF- κ B and Comp1 signaling to increase inflammation require further investigation.

Other genes whose expression was modulated by Erb2 that may be involved in the inflammatory response include *Ptprc*. The expression of *Ptprc*, which is involved in the migration of inflammatory cells after UV exposure,^{46–48} was decreased on inhibition of Erb2. Many UV-regulated cytokines that activate or chemoattract inflammatory cells such as *Ccl12*, *Ccl11*, and *Cxcl2* were regulated by Erb2. *Ccl11*, in particular, induces the migration of Cd53-positive mast cells to the skin.⁴⁹ *Cd53* is up-regulated to protect against oxidative stress and UVB. Although mast cells are best known for their role in allergic reactions, they are also important in the response to stress, and are induced in the skin after UV exposure (reviewed in Ref. 50). Thus, Erb2 may induce mast cell migration by increasing *Ccl11* expression. Changes in the expression of inflammation-related genes may also occur through an indirect mechanism involving cellular changes that alter the response of the stroma or tissue infiltrate. Analysis of the expression of cytokines and other inflammatory gene products in skin and cultured keratinocytes lacking Erb2 activity is underway to distinguish between these possibilities.

The skin contains many defenses for protection from environmental insults and oxidative stress. The generation of ROS by UV is met by a quick response of increasing both detoxifying enzymes and inflammatory mediators to maintain normal homeostasis. The response is partly due to the activation of redox-sensitive transcription factors.⁵¹ However, UV-generated ROS also result in the activation of Erb2, which as our results demonstrate, increases inflammation. These data indicate that a portion of the inflammation caused by UV-induced ROS occurs indirectly as a result of Erb2 activation.

Inhibition of Erb2 altered the expression of many proliferation-associated genes after UV as well. The sharp decline in cyclin expression 6 hours after UV exposure was consistent with cell cycle arrest after UV. The skin, however, must maintain its integrity and replenish lost cells; this is illustrated by the rebound of cyclin gene expression and BrdU incorporation 24 hours after UV

exposure. *Tk1* expression, which is involved in the synthesis of DNA and is a marker of cell proliferation,⁵² followed the same pattern of gene expression demonstrated by the cyclins in our experiments. Inhibition of *ErbB2*, however, altered the pattern of expression of these genes such that their decrease in expression at 6 hours after UV was not as great but neither was the recovery of expression at 24 hours. In some cases, the expression of the genes continued to decrease at 24 hours after UV exposure. These data suggest that *ErbB2* deregulates cell cycle arrest after UV exposure as well as the recovery of proliferation at later times. Indeed, our data showed that *ErbB2* is necessary for keratinocyte proliferation after UV exposure. The mechanisms by which *ErbB2* modulates the expression of cell cycle genes and cell cycle progression warrant further investigation.

Although not as dramatic of an effect as on inflammation or proliferation, abrogation of *ErbB2* also reduced survival after UV and regulated apoptosis-associated genes. This may be the result of increased expression of proapoptotic genes like *Pawr*⁵³ and *Sox4*⁵⁴ after UV. In addition, *Pdcd4* expression, which is induced during apoptosis⁵⁵ and induced by the *ErbB2* antagonist Herceptin in breast cancer cells,⁵⁶ was similarly increased. The role of *ErbB2* in promoting cell survival may also occur through increased antiapoptotic gene expression, such as *Ghr*⁵⁷ and *Ptgs2*, which has been shown to promote survival by decreasing apoptosis after UV exposure to keratinocytes.⁵⁸ Consistent with these data, cell culture experiments to validate the microarray analysis demonstrated that inhibition or knockdown of *ErbB2* increased apoptosis slightly but significantly after UV, implying *ErbB2* enhances cell survival after UV. However, the biological significance of an effect of this magnitude remains unclear. Although the number of apoptosis genes with increased expression on *ErbB2* inhibition was similar at 6 and 24 hours after UV, an increase in apoptosis was not detected until 24 hours, most likely reflecting the time required for protein synthesis and proapoptotic signaling to manifest in DNA cleavage.

Our microarray analysis and cell culture experiments revealed that inhibition of *ErbB2* decreased proliferation, increased apoptosis, and suppressed inflammation after UV irradiation, all processes intimately linked to cancer development and progression. Fifty-four genes regulated by *ErbB2* were linked to cancer in the literature, many of these specifically associated with skin cancer. For example, *Ptgs2* expression is increased in many cancers including skin cancer,⁵⁹ and *Hif1a* is overexpressed in squamous cell head and neck cancer, correlated with aggressive behavior and resistance to chemotherapy.⁶⁰ Inhibition of *ErbB2* reduced the expression of *Map3k8*, a proto-oncogene that is known to act simultaneously on all known MAPK cascades,⁶¹ consistent with the activation of MAPK signaling in head and neck squamous cell carcinoma.⁶² *ErbB2* also activates NF- κ B, which is increasingly recognized as important in cancer (reviewed in Ref. 63) and is constitutively activated in head and neck squamous cell carcinoma.⁶⁴ Although the link between inflammation and cancer is complex (reviewed in

Ref. 65), changes in inflammatory gene expression have been demonstrated in basal cell carcinoma.⁶⁶ *Ghr* expression, suppressed on inhibition of *ErbB2*, is a marker for the progression from actinic keratosis to squamous cell carcinoma.⁶⁷ Significant correlations have been shown between *Mmp9*, reduced by the *ErbB2* inhibitor, and *ErbB2* expression with respect to clinico-pathological parameters in head and neck squamous cell carcinoma,⁶⁸ and oral squamous cell carcinomas have higher expression of *Mmp9*.⁶⁹ These data support a multifaceted role for *ErbB2* in skin cancer development and progression.

Our analysis revealed that *ErbB2* not only activated NF- κ B but also Comp1, FoxJ2, and Tal1. Although not much is known about Comp1 and FoxJ2 specifically, deregulation of Fox family members is involved in carcinogenesis (reviewed in Ref. 70) and inflammation.⁷¹ Tal1 has a role in hematopoiesis, migration and angiogenesis.⁷² Transgenic expression of Tal1 can cause malignancies,⁷³ and its loss can induce apoptosis.⁷⁴ Further investigation of *ErbB2*-regulated transcription factors is needed.

As documented previously,^{3,5,9,13,75} UV exposure resulted in the activation and up-regulation of EGFR (Figures 1B and 8B). *ErbB2* receptor expression also increased after UV exposure although there is some variation in this response (Figure 1B, Figure 8B, and data not shown). Thus, both increased phosphorylation and increased expression may contribute to *ErbB2*'s effects. Interestingly, our data revealed that inhibition or siRNA knockdown of *ErbB2* before UV exposure also prevented the phosphorylation of EGFR-Tyr⁹⁹² and *ErbB3*-Tyr¹²⁸⁹ (Figures 1B and 8B). This implies that *ErbB2* heterodimerizes with each of these receptors in response to UV irradiation. The immunoblot of phospho-EGFR(Tyr¹¹⁷³) likewise demonstrates UV-induced phosphorylation at this site, as was expected from previous studies,¹³ however, this activation was not decreased by inhibition of *ErbB2* (Figure 1B). This information suggests that an examination of *ErbB2* heterodimerization and receptor phosphorylation following UV exposure may reveal the activation of specific signaling pathways. Preferential dimerization partners of *ErbB2*, their activation sites, specific signal transduction pathways invoked, and their role in the response of the skin to UV warrant further investigation.

In summary, we have identified many novel genes regulated by *ErbB2*, demonstrating its importance in the response of skin to UV. Our data demonstrate that *ErbB2* activation after UV exposure increases inflammation, increases proliferation, and suppresses apoptosis. The mechanisms through which *ErbB2* causes inflammation through NF- κ B, Comp1, and possibly FoxJ2 require further investigation. These data also implicate *ErbB2* in UV-induced skin cancer. Although the role of *ErbB2* in tumorigenesis in other tissues is well documented, little data exist about the role of *ErbB2* in nonmelanoma skin carcinoma. This research supports further investigation into the role of *ErbB2* in UV-induced inflammation and skin cancer.

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