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ORIGINAL ARTICLE

Transcriptional effects of inhibiting epidermal growth factor receptor in keratinocytes

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ABSTRACT

Background: Epidermal growth factor receptor (EGFR) activation is important in human epithelial malignancies, including cutaneous squamous cell carcinoma, lung, colon, pancreatic, and other cancers. Therapies targeting EGFR are currently used to treat such cancers, but one significant drawback to EGFR inhibitor therapies is the associated skin toxicity. This toxicity usually presents as papular or pustular folliculitis, dry skin with pruritus and hair and nail abnormalities. The side effects often limit the usefulness of EGFR inhibitors in cancer treatment. The transcriptional changes caused by EGFR inhibition in epidermal keratinocytes have not been extensively explored.

Methods: To define the transcriptional changes caused by inhibition of EGFR in primary human epidermal keratinocytes, these cells were treated with Tyrphostin AG1478 and treated and control cultures were compared in parallel, using Affymetrix microarrays. Using meta analysis approaches, the observed changes were integrated with a large set of already existing data on transcriptional profiling in epidermal keratinocytes.

Results: We found that at the early time points, 1 hour and 4 hours after addition, AG1478 suppresses expression of genes associated with keratinocyte proliferation, attachment and motility. Apoptosis is facilitated by both induction of proapoptotic and suppression of antiapoptotic genes. Angiogenesis signals are suppressed as well. At late time points, 24 hours and 48 hours, EGFR inhibition induces mitochondrial activity and suppresses splicing and protein trafficking. Certain transcriptional effects of EGFR inhibition go against the transcriptional effects of retinoids. Surprisingly, at 48 hours, EGFR inhi bition induces expression of markers of epidermal differentiation.

Conclusion: Our results define the role of EGF receptor in human keratinocytes and the consequences of its inhibition.

Introduction

Among the first signaling molecules isolated, epidermal growth factor (EGF) was named for its capacity to accelerate epidermal differentiation, as measured by the eye opening in newborn mice.[1] Since then, EGF and its receptor (EGFR) have become possibly the most studied models of extracellular and intracellular signaling mechanisms.[2 5] The binding of EGF to its receptor causes the receptor to dimerize and consequently activate its cytoplasmic kinase.[6] The activated kinase phosphorylates several substrates which, when phosphorylated, transmit cytoplasmic signals to the nucleus by activating transcription factors that regulate expression of many genes.[7] In general, the EGF regulated genes promote cell proliferation and survival, inhibit apoptosis, support chemotactic migration, while suppressing terminal differentiation.[8] Several ligands in addition to EGF bind and cause activation of EGFR, e.g., tumor growth factor α (TGFα), and heparin binding EGF.[9] EGFR is closely related to the ErbB family of protein kinases.[10]

EGFR is very important in healthy epidermal homeostasis and disruption of the EGFR signaling has been demonstrated in several skin diseases.[11] In epidermis, EGFR activation contributes to wound healing, regulates barrier function, suppresses terminal differenti ation, causes loss of adhesion, induces secreted proteases, etc.[11,12] Furthermore, EGFR is often over expressed in cutaneous squa mous cell carcinomas.[13]

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EGFR activation is a feature of numerous human epithelial malignancies, including cancers of the colon, head and neck, lung, and pancreas.[8] Therefore, therapies targeting EGFR by small molecules or antibodies became a paradigm for treatment of human cancers.[3] Detailed molecular understanding of the kinase domain led to development of specific inhibitors, gefitinib, erloti nib, Herceptin (trastuzumab), cetuximab, currently used to treat breast, lung, ovarian, prostate, head and neck, and other cancers.[8,14] These drugs induce tumor regressions proving the importance of the EGFR signals in some cancers. EGFR targeted treatments avoid the adverse effects commonly encountered with chemotherapy, such as nausea, vomiting or hematological toxicities. However, there are two significant drawbacks to EGFR inhibitor therapies: (1) the intrinsic and acquired resistance to treatment; and (2) the associated skin toxicity.[15,16] The cutaneous reactions to these treatments usually present as papular or pustular folliculitis erup tions. Later manifestations include generalized dry skin, pruritus, and hair and nail abnormalities.[17] These side effects often limit the duration or dosage of EGFR inhibitors in cancer treatment.

Given the importance of cutaneous manifestations of EGFR targeted therapies, it is surprising that the transcriptional changes caused by the EGFR inhibition have not been extensively explored in epidermal keratinocytes. To define the transcriptional changes caused by inhibition of EGFR in primary human epidermal kerati nocytes, we treated these cells with Tyrphostin AG1478, a specific inhibitor of the EGFR kinase domain, and compared parallel treated and control cultures using Affymetrix microarrays. We used publi cally available, free meta analysis programs to integrate the observed changes with a large set of already existing data on transcriptional profiling in epidermal keratinocytes.[18–23] We found that Tyrphostin AG1478 inhibits expression of genes associated with keratinocyte proliferation, attachment and motility and, surpris ingly, induces expression of markers of epidermal differentiation.

Materials and methods

Human keratinocyte cultures

The growth and treatment of normal human neonatal foreskin epidermal keratinocytes has been described.[18,24,25] Briefly, keratinocytes were obtained from Dr. M. Simon (Living Skin Bank, Burn Unit SUNY, Stony Brook, NY, USA) and grown in a defined serum free keratinocyte growth medium (keratino cyte SFM; Gibco, Grand Island, NY, USA) supplemented with 2.5 ng/mL epidermal growth factor, 0.05 mg/mL bovine pitui tary extract, and 1% penicillin/streptomycin at 37°C in 5% CO_2. Third passage cells were used at 70–80% confluency. Kerati nocytes, grown on 100 mm plates, were then treated with 1μM Tyrphostin AG1478 (Sigma Aldrich, St Louis, MO, USA). The cells were harvested by scraping 1 hour, 4 hours, 24 hours and 48 hours after treatment.

Preparation of labeled cRNA and GeneChip hybridization

Total RNA was isolated from treated and untreated keratinocytes with RNeasy kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Approximately 5 μg of total RNA was reverse transcribed, amplified, and labeled as described previ ously.[26,27] A 15 μg sample of labeled cRNA was fragmented and hybridized to HU133Av2 arrays (Affymetrix, Santa Clara, CA, USA). The arrays were washed, stained with anti biotin streptavidin phycoerythrin labeled antibody, and scanned using the Agilent GeneArray scanner system (Hewlett Packard, Palo Alto, CA, USA) and GeneChip 3.0 software (Affymetrix) to determine the expres sion of each gene.

Array data analysis

Generally, the same data analysis approach as described previously was used.[24] Intensity values from the chips were obtained using Microarray Suite v. 5.0 (Affymetrix), and scaled by calculating the overall signal for each array. To compare data from multiple arrays, the signal of each probe array was scaled to the same target intensity value of 500 arbitrary units. RMAExpress was used for background adjustment, quintile normalization, summarization, and quality analysis. RMAExpress is a program that computes gene expression summary values and performs quality assessment for Affymetrix data using the Robust Multichip Average protocol.[28] Raw data CEL files have been deposited in the Gene Expression Omnibus (GEO) database and are available with the accession number GSE41680. Regulated genes were selected as differentially expressed 50% or more between the Tyrphostin AG1478 treated and the control samples at the same time point. The hierarchical clustering was performed using TIGR MultiExperiment Viewer algorithms.[29] Annotation and ontology of regulated genes was ob tained using Database for Annotation, Visualization and Integrated Discovery (DAVID).[30,31] Transcription factor binding sites were also evaluated using DAVID, in a separate analysis. In addition, lists of Affymetrix IDs of regulated genes were submitted to DAVID.[30] DAVID provided 'tables' containing functional and ontological details of the regulated genes, 'charts' containing ontological cate gories, pathways etc., over represented in the gene lists, 'clusters' of such ontological categories (which identified redundancies and overlaps), transcription factors over represented in the promoters of the genes, as well as sub lists of genes specific for each onto logical category. From the 'tables', lists of consensus gene symbols were re formatted, and submitted to the List2Networks program[32]. This allowed identification of specific functional commonalities in various lists of genes, providing the meta analysis results.

Meta-analysis comparisons of regulated genes in keratinocytes

In our previous studies we defined the genes regulated in kerati nocytes by interferon γ (IFNγ), interleukin 1 (IL 1), retinoic acid (RA), EphrinA proteins, and JNK inhibitor SP600125.[18,19,21,23,25] Using DAVID, the lists of official symbols of regulated genes in those studies were collected, as were those of the Tyrphostin AG1478 regulated genes from this study, and these were then submitted to the List2 Network analysis program.[32] The program compares lists for mutual overlaps within specific categories, e.g., targets of protein kinases, ontological biological process, or OMIM disease gene association, returning statistical evaluation of the overlaps. When seven different relevant categories were analyzed, it was found that 'Gene Ontology Biological Process' is the most informative, probably because it is the best annotated and most complete. The matrices of *p* values of gene lists overlaps were downloaded, along with spreadsheets of *p* values of individual biological processes, Bonferroni corrected for multiple comparisons.

Results

Induced genes

The complete list of induced and suppressed genes is given in Supplement Tables 1 and 2. They are too numerous to display in the text. In addition, the raw data values were deposited in the GEO database (GSE41680).

In the first 1–4 hours after the addition of Tyrphostin AG1478, perhaps expectedly it being an inhibitor of EGFR signaling, expression of more genes was suppressed than is induced. Espe cially at the first time point, 1 hour, while many categories were