Allergens and Irritants Transcriptionally Upregulate CD80 Gene Expression in Human Keratinocytes

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The human CD80 costimulatory molecule is an important signal between professional antigen-presenting cells and T helper cells. The immunobiology of CD80 expression by keratinocytes, especially during allergic and irritant contact dermatitis, however, is less well understood. CD80 cell surface expression and gene transcription by keratinocytes was increased when keratinocytes were exposed to certain allergens (chemicals that induce inflammation via hapten-specific T cells) and irritants (chemicals that are toxic to epidermal cells). Therefore, the human CD80 promoter was cloned and luciferase reporter constructs containing various promoter fragments were engineered. Promoter mapping of these CD80 constructs in transiently transfected keratinocytes showed that a construct containing the proximal 231 bp immediately upstream of the transcription start site of the CD80 promoter was most active in keratinocytes and was inducible to a level ranging from 2- to 10-fold higher in keratinocytes treated with certain allergens and irritants, compared with untreated keratinocytes. This pattern of promoter fragment activity in keratinocytes is identical to that found in professional antigen-presenting cells. This is the first demonstration that the CD80 promoter is active in keratinocytes and that this activity is further increased in keratinocytes treated with certain allergens and irritants. These data suggest that allergens and irritants may, in part, break peripheral tolerance by their direct effects on keratinocyte costimulatory molecule expression, thereby facilitating interactions with epidermotropic T helper cells via the CD80–CD28 or CTLA-4 pathways. *Key words: costimulatory molecules/irritant and allergic contact dermatitis/keratinocytes/promoter. J Invest Dermatol 114:1085–1092, 2000*

B oth irritant contact dermatitis and allergic contact dermatitis (ACD) are common and important conditions in dermatology and occupational medicine. Comparative studies of irritant contact dermatitis and ACD in humans and animal model systems indicate that whereas their clinical definitions are separate and distinct, these two types of skin inflammation have many overlapping features indicating that they share many common effector pathways (Gaspari, 1997). Irritant contact dermatitis is a nonimmunologic, local inflammatory reaction following a single or repeated exposure to an irritant (a chemical that is toxic to epidermal cells) (Mathias, 1978). Evidence, however, indicates that the immune system is an important effector mechanism for mediating the skin inflammation triggered by a toxic insult. In contrast, ACD is a prototypical, cutaneous delayed-type hypersensitivity reaction (type IV allergy), following exposure to a hapten. ACD is dependent on epidermal Langerhans cells presenting haptenated self proteins to CD4+ T lymphocytes in regional lymph nodes in both the afferent (primary, inductive phase) and the efferent (secondary, effector phase) arms of contact hypersensitivity (Kalish, 1978; Gaspari, 1993).

T cell activation by Langerhans cells or other professional antigen–presenting cells (APC) requires two signals. The first signal involves the interaction of major histocompatibility complex (MHC) bound antigen with its cognate T cell receptor. This is followed by a secondary signal between a costimulatory molecule and its specific receptor on the T cell (Mueller *et al*, 1989). Several costimulatory molecules which can deliver this secondary signal have been described and include the two members of the B7 family, CD80 (B7-1) and CD86 (B7-2) (Dustin *et al*, 1989; Van-Seventer *et al*, 1990; Gimmi *et al*, 1991; Linsley *et al*, 1991a; Armitage *et al*, 1992; Liu *et al*, 1992; Young *et al*, 1992). The corresponding T cell receptors for the B7 costimulatory molecules are CD28 and CTLA-4 (CD152) (Thompson *et al*, 1989; June, 1990; Linsley *et al*, 1990, 1991b). Antigen presentation plus the secondary costimulatory signal by APC results in the successful activation of T cells, and autocrine growth factor production. Antigen presentation, however, in the absence of a secondary costimulatory signal leads to T helper cell clonal anergy, a type of immunologic unresponsiveness (Mueller et al, 1989).

CD80 and CD86 costimulatory molecule expression by professional APC, including dendritic cells, monocytes, and activated B cells, is a tightly regulated process. CD86 is constitutively expressed on resting monocytes (Azuma et al, 1993); however, most other APC require a stimulus for the induction of both CD80 and CD86. These stimuli can include certain cytokines [interferon (IFN-γ)],

cyclic adenosine monophosphate, cross-linking of MHC class II molecules, CD40 or CD120b (p75, tumor necrosis factor receptor-II) (Freedman *et al*, 1991; Ranheim and Kipps, 1993) and certain bacteria or their products, including BCG (Thurnher *et al*, 1997), cholera toxin A1 subunit (Agren *et al*, 1997), and neisserial porins (Wetzler *et al*, 1996). The kinetics of induction of cell surface expression are different for CD80 and CD86 gene expression. In activated B cells, CD86 gene expression is induced first, at 24 h following activation, whereas CD80 is not induced until 48 h postactivation (Lenschow *et al*, 1992; Boussiotis *et al*, 1993).

Keratinocytes (KC) are nonfunctional epidermal APC in normal skin. Previous studies of *in vitro* antigen presentation by Ia$^+$ KC indicate that they do not activate antigen-specific T helper cell proliferation (Gaspari *et al*, 1988), instead, they induce clonal anergy (Gaspari *et al*, 1988; Bal *et al*, 1990). Using mouse model systems of *in vivo* antigen presentation for contact hypersensitivity, Ia$^+$ KC are also tolerogenic APC (Gaspari and Katz, 1991). This type of tolerogenic antigen presentation by epidermal KC fits well with the two-signal model of T helper lymphocyte activation (Mueller *et al*, 1989). When B7$^-$ KC-derived cell lines are induced to express significant levels of cell surface CD80 by DNA-mediated gene transfection, followed by IFN-γ treatment to induce cell surface expression of HLA-DR, such transduced KC can induce T helper lymphocyte proliferation in a mixed lymphocyte reaction (Gaspari, 1993).

Relatively little is known about CD80 and CD86 gene expression by nonbone marrow-derived cells such as KC. Normal human KC have been reported to either not express cell surface CD80 (Simon *et al*, 1994) or express it at a low basal level (Fleming *et al*, 1993; Vandenberghe *et al*, 1993; Nasir *et al*, 1995), which is insufficient as a secondary, costimulatory signal and therefore results in T cell anergy or tolerance under normal conditions. In T cell-dependent inflammatory skin disease states, however, *in situ* immunohistochemical studies of these affected tissues revealed increased levels of cell surface CD80 molecules by KC (Nickoloff *et al*, 1993; Simon *et al*, 1994). As the inflammatory infiltrate in these conditions is predominantly comprised of T lymphocytes, it has been hypothesized that this increased expression of CD80 may play a part in amplifying the T cell-mediated hypersensitivity reactions seen in these skin conditions.

In this report, the role of both CD80 transcriptional regulation and cell surface expression in human KC exposed to certain allergens and irritants has been studied. Cell surface expression of CD80 by KC is increased *in vivo* during ACD in positive patch test reactions. Gene transcription (nuclear run-off analysis) of CD80 is greatly increased in KC cultured in the presence of a model allergen, nickel chloride (NiCl$_2$), or a model irritant, sodium lauryl sulfate. Finally, when the luciferase reporter gene was placed under transcriptional control of fragments isolated from the CD80 promoter, this promoter was observed to be active at a low level in KC and was significantly induced by exposure to various allergens and irritants. Taken together, these data indicate that KC transcriptionally regulate CD80 similar to professional APC, and that chemicals causing contact dermatitis upregulate this expression, which may contribute to the immunopathology of this common dermatologic condition.

MATERIALS AND METHODS

Cells and media Human KC and fibroblasts, both isolated from neonatal foreskin (Gaspari *et al*, 1996) and Raji cells (Burkitt lymphoma B cell, ATCC, Rockville, MD) were used in these experiments. KC were cultured in serum free Medium 154CF (Cascade Biologics, Portland, OR) supplemented with human KC growth supplement (1×), gentamicin (50 µg per ml) and calcium chloride (50 µM) unless otherwise noted. Both Raji and fibroblasts were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), penicillin G (100 units per ml), streptomycin (100 µg per ml) and amphotericin B (250 ng per ml).

Immunohistochemical analysis of CD80 on ACD skin biopsy specimens Trephine biopsies were taken from untreated skin and positive (2 + reactions) 48 h patch tests to NiSO$_4$, fixed in 10% formalin and embedded in paraffin blocks. Transverse sections (5 µm thick) were cut on to glass microscope slides. The sections were incubated with 10 µg per ml BB-1, a monoclonal anti-CD80 antibody (PharMingen, San Diego, CA), followed by second step antibodies from the Vectastain ABC anti-mouse IgM Glucose Oxidase Kit (Vector Laboratories, Burlingame, CA) Samples were developed using the Vecta Glucose Oxidase Kit III.

Skin allergy testing for ACD (patch testing) Patients with suspected ACD were patch tested using standard methods with a panel of allergens from North American Series of chemical allergens (Hermal Pharmaceutical Labs, Oak Hill, NY) applied to the skin of the back with Finn chamber occlusion (Adams *et al*, 1990). After 48 h, the patches were removed and graded using a standard grading system (Adams *et al*, 1990). After obtaining informed consent, skin biopsy specimens were obtained from normal skin and from strong positive patch test reactions.

KC adherent cell staining Primary culture KC were grown on coverslips and were either not treated (medium alone), treated with phorbol-12-myristate-13-acetate (PMA) (25 ng per ml) or treated with IFN-γ (500 units per ml) for 48 h. Cells were stained with either 20 µg per ml anti-human CD80 (BB-1) or 20 µg per ml anti-human CD74 (PharMingen) followed by fluorescein isothiocyanate (FITC)-goat anti-mouse IgM (10 µg per ml for BB-1) or FITC-goat anti-mouse IgG (10 µg per ml for CD74) (BioSource, Camarillo, CA), respectively. Stained cells were visualized on an Eclipse E800 Florescent microscope (Nikon, Japan). Raji cells were used as a CD80 positive control (Sempowski *et al*, 1997).

Analysis of adherent KC CD80 and CD74 expression by confocal scanning laser microscopy (CSLC) The level of cell surface CD80 and CD74 expression by cultured KC that were adherent to sterile glass coverslips was analyzed by fluorescence-based CSLC with a Meridian Ultima Adherent Cell Analysis System (ACAS) and its accompanying Data Analysis System (DASY) Master Program, vs. 3.32 (Meridian Instruments, Okemos, MI). CSLC with the ACAS and DASY provided imaging capacity on stationary samples comparable with conventional CSLC plus the added quantitative analysis of fluorescence intensity provided by flow cytometry but without potential cell damage (trypsinization) which increases nonspecific staining. Samples were excited with a 488 nm argon laser line, and emissions below 575 nm were detected with a photomultiplier tube. All samples were examined with a 20× objective and scanned with an identical set of parameters so that results from treatment conditions to the KC could be compared. In all cases, the laser beam (approximately 0.3 µm in thickness) scanned the sample in a Raster pattern at intervals of 1.0 µm. Fluorescence was detected from the cell surface of the KC over an area of approximately 2500 mm².

From the data points collected, digitalized images of the scanned sections were generated that corresponded to a scale of pixel values ranging from 3 to 17,310 relative fluorescent units (RFU). From the digitalized images, statistical analysis was performed with the DASY software package. These included (i) the number of fluorescent pixels in the scanned area above the background threshold area; (ii) the average fluorescence in RFU, which was obtained by dividing the total integrated value of the foreground RFU by the number of data points collected; and (iii) the average background threshold level. In the images derived from the stained KC, pixels with relative fluorescence values of 100 or less were subtracted as background from the original scan prior to the statistical analysis.

Polymerase chain reaction (PCR) amplification and cloning of the CD80 promoter Two groups have previously reported the cloning of the human CD80 promoter and characterized this promoter's activity in Raji cells (B cell line) (Fong *et al*, 1996; Zhao *et al*, 1996). In order to characterize the activity of the CD80 promoter in KC, we have PCR amplified a 3875 bp segment of the human CD80 gene promoter using primers generated from a published sequence (EMBL/GenBank/DDBJ accession number U33208). The CD80 promoter fragments were cloned into either pGL3-basic or pGL3-enhancer (SV40 enhancer) vectors. The positive control vectors used were pGL3-control with an SV40 enhancer and promoter or pRL-CMV with a CMV promoter and enhancer (Promega, Corp., Madison, WI). PCR products were amplified using the Expand Long Template PCR System (Boehringer Mannheim, Indianapolis, IN), dNTPs (500 µM), primers (300 µM), human genomic DNA (250 ng), and Expand Long DNA polymerase (0.5 µl) in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). Amplification was

for 30 cycles of 94°C for 10 s, 58°C for 10 s, and 72°C for 3 min, with a 20 s elongation extension for the last 20 cycles.

We amplified the CD80 promoter using a 26-mer 5′ primer extending from nucleotide −3426 to nucleotide −3401 (AAAGAGTCACAGG-CAGGAAGAGTAAG), whereas the 3′ primer was a 25-mer extending from nucleotide +449 to nucleotide +425 of the CD80 gene (ACTGGCTTGAGAAATTCAAATACGG). The 3875 bp CD80 PCR product was cut with Bam HI to produce a 3709-bp fragment which was cloned into the SmaI and BglII sites of pGL3-Basic (pB7/3709) and Enhancer (pB7e/3709) luciferase reporter vectors (**Figs 1 and 5**). A smaller fragment of 533 bp was also PCR amplified using a 5′ primer (23-mer), extending from nucleotide −84 to nucleotide −61 (AGCAAGTAGAA-GAAGACGGCAGC) and the same 3′ primer described above. Similarly, this DNA was cut with *Bam*HI producing a 367 bp fragment, which was also cloned into the *Sma*I and *Bgl*II sites of the luciferase reporter vectors (pB7/367).

Three other fragments of varying lengths were cut from the 3875 bp PCR product using the restriction enzymes *Nhe*I, *Eco*RI, or *Hin*dIII in conjunction with *Bam*HI to produce the following fragments: 514 bp extending from −231 to +283; 760 bp extending from −467 to +283; and 1132 bp extending from −849 to +283. The 514 bp piece was cloned into the *Nhe*I and *Bgl*II sites of pGL3 reporter vectors (pB7/514) and the 760 and 1132 bp pieces were cloned into reporter vectors by ligating the *Bam*HI cut end into the vector's *Bgl*II site and blunt end ligating the *Eco*RI and *Hin*dIII sites into the *Sma*I site of the vector (pB7/750 and pB7/1132). These 5 CD80 promoter constructs (**Fig 1**) approximate the previously published CD80 promoter constructs (Fong *et al*, 1996; Zhao *et al*, 1996).

Transient transfection and luciferase assays Raji (1×10^5 cells), human KC (90% confluent) and human fibroblasts (90% confluent) were transfected in 96 well tissue culture plates using 50–500 ng lipofectin (Gibco BRL) and 0.1–1.0 μg per well DNA in a volume of 100 μl of either RPMI minus fetal bovine serum (Raji and fibroblasts) or 154CF (KC) (Strauss, 1996). After 4–18 h, 100 μl fresh RPMI plus 20% fetal bovine serum or 154CF was added and at 48 h the cells were lyzed and assayed for luciferase expression using either the Luciferase Assay System or Dual-luciferase reporter assay system (Promega) and a Microlumat LB96P luminometer (Groskreutz *et al*, 1996). (EG & G Berthold, Germany) Cell lysate protein concentrations were determined by Bradford assay at OD 595 nm using a Benchmark microplate reader (Bio-Rad, Richmond, CA). Luciferase expression is reported as relative luminescence units (rlu) per μg protein.
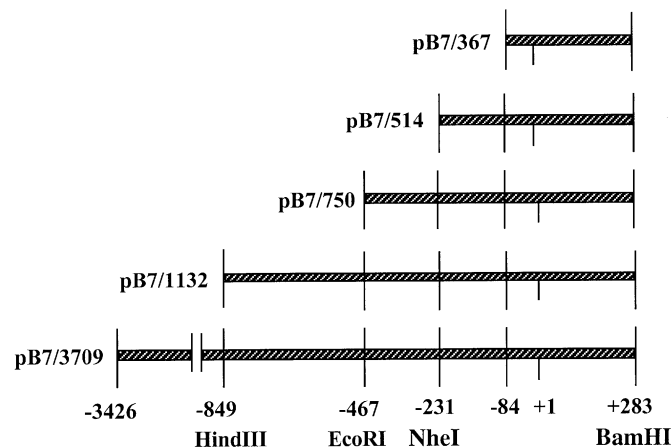

Figure 1. CD80 promoter clones. Five CD80 promoter clones were constructed and used for the transient transfection of KC and other cell lines. The clones were ligated into pGL3-basic (pGL3) or pGL3-enhancer (pGL3e) luciferase reporter vectors. Vertical lines denote the nucleotide position of the CD80 fragments in each construct along with the important restriction enzyme sites used in their cloning. The CD80 nucleotide position (+1 signifies the CD80 transcription start site) and restriction sites are noted along the bottom of the figure. Each construct contains the first 283 bp (+1 to +283) of the transcribed region of the CD80 gene linked to the firefly luciferase gene. The constructs, from the shortest to longest contain 84, 231, 467, 849, and 3426 bp of the untranscribed CD80 promoter region, respectively.

Statistical analysis of gene reporter assays All gene reporter assays were analyzed for statistical significance using the one-tailed Student t test (Statview Software, Abacus Concepts, Berkeley, CA). $p < 0.05$ were considered statistically significant.

RESULTS

Increased CD80 KC cell surface expression in skin involved in ACD Immunohistochemical analysis of CD80 (BB-1) cell surface expression in sections from skin biopsy specimens taken from normal skin revealed rare stained cells (**Fig 2a**). In contrast, a (2+) positive patch test for NiSO$_4$ (a common allergen often found in jewelry) (Peltonen, 1979) showed a dramatic increase in CD80 expression (**Fig 2b**) compared with normal skin. The CD80 staining was distributed evenly throughout the epidermis and involved predominantly KC. Our immunochemistry studies of skin biopsy specimens of ACD due to patch testing with a variety of allergens in seven other patients (NiSO$_4$, potassium dichromate, quaternium 15, formaldehyde resin, and colophony) revealed a similar pattern of CD80 by KC (data not shown). This clearly indicates that KC from skin involved in ACD increase their cell surface expression of CD80.

Differential cell surface expression of CD80 and CD74 in KC treated with PMA or IFN-γ It has been reported that the monoclonal antibodies (MoAb) BB-1 binds not only CD80 but also CD74 on B cell precursor cells (Freeman *et al*, 1998) and that BB-1 binding on these cell surfaces indicates the total of both CD80 and CD74 expression. This group, however, did not examine cell surface expression of BB-1 and CD74 in KC. Therefore, we compared BB-1 and CD74 cell surface expression in human KC treated with IFN-γ or PMA for 48 h, then incubated with either BB-1 or an anti-CD74 MoAb followed by FITC-labeled secondary antibodies (**Fig 3**). IFN-γ-treated KC showed only a slight increase in BB-1 binding whereas PMA-treated KC showed a large increase in BB-1 binding compared with untreated cells (**Fig 3a**). In contrast, the binding of anti-CD74 MoAb was greatly increased on IFN-γ-treated KC but did not bind to PMA-treated cells compared with untreated KC (**Fig 3b**). These stained KC were then analyzed by CSLC, and the relative levels of fluorescence from each specimen was quantitated. This quantitative analysis of the pattern of CD80 and CD74 expression indicates the differential pattern of expression of the two different antigens (**Table I**). These results suggest that the BB-1 cell surface binding on KC is not due to the expression of CD74, instead, and may represent CD80 expression. Cell-specific activation of CD80 promoter by Raji cells and human fibroblasts with CD80 luciferase reporter vectors. We transfected the five promoter constructs, pB7e/367 (84 bp of the CD80 promoter), pB7e/514 (231 bp of the CD80 promoter), pB7e/750 (467 bp of the CD80 promoter), pB7e/1132 (849 bp of the CD80 promoter), and pB7e/3709 (3426 bp of the CD80 promoter) (see **Fig 1**) into Raji (CD80 positive B cell line) and fibroblasts (CD80 negative) (Sempowski *et al*, 1997) to measure the cell-specific transcriptional activity of these promoter fragments (**Fig 4**). Our promoter constructs (containing an SV40 enhancer), show cell-specific activity (3–5-fold increase in activity compared with the pGL3-enhancer vector alone) in Raji cells, but not in human fibroblasts (**Fig 4**). Transfection of both Raji and fibroblasts using the positive control vectors pGL3-control or pRL-CMV, gave equivalent, high levels of luciferase expression (data not shown). Another group (Fong *et al*, 1996) have also reported cell-specific activity for similar CD80 promoter constructs containing 84 and 253 bp fragments (but not longer fragments) from the 3′ end of the CD80 promoter that were transcriptionally active in CD80$^+$ cell lines, but not in CD80$^-$ cell lines when transfected with a vector containing an SV40 enhancer.

Our data indicate that CD80 promoter activity in cultured cells correlates well with CD80 cell surface expression. That is, cells that express cell surface CD80 (Raji cells) demonstrate CD80 promoter
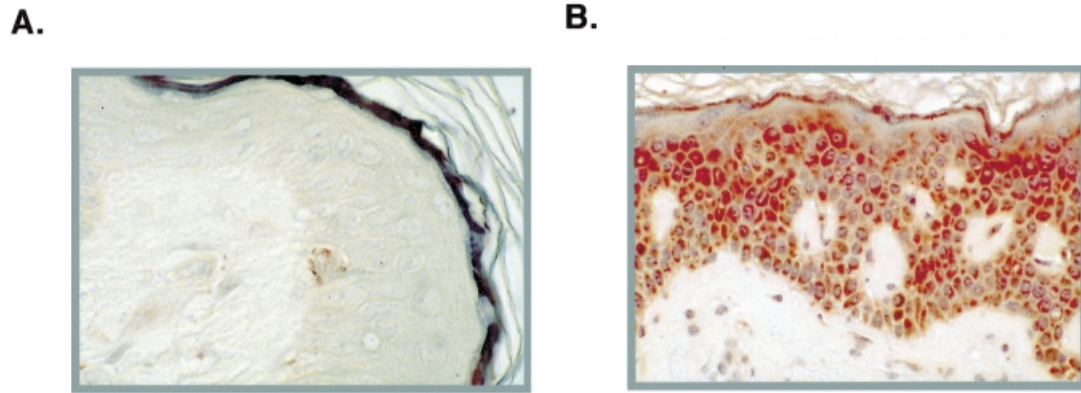
Figure 2. Immunohistochemical analysis of CD80 cell surface expression in skin involved in ACD. Sections from skin biopsy specimens taken from (*A*) untreated, normal skin (40× magnification) and (*B*) positive (2+) patch test to NiSO$_4$ (40× magnification). Sections were incubated with MoAb, BB-1, followed by secondary antibodies from the Vectastain ABC anti-mouse IgM Glucose Oxidase Kit. Staining with the secondary antibody alone did not result in any staining of the tissue sections (not shown).
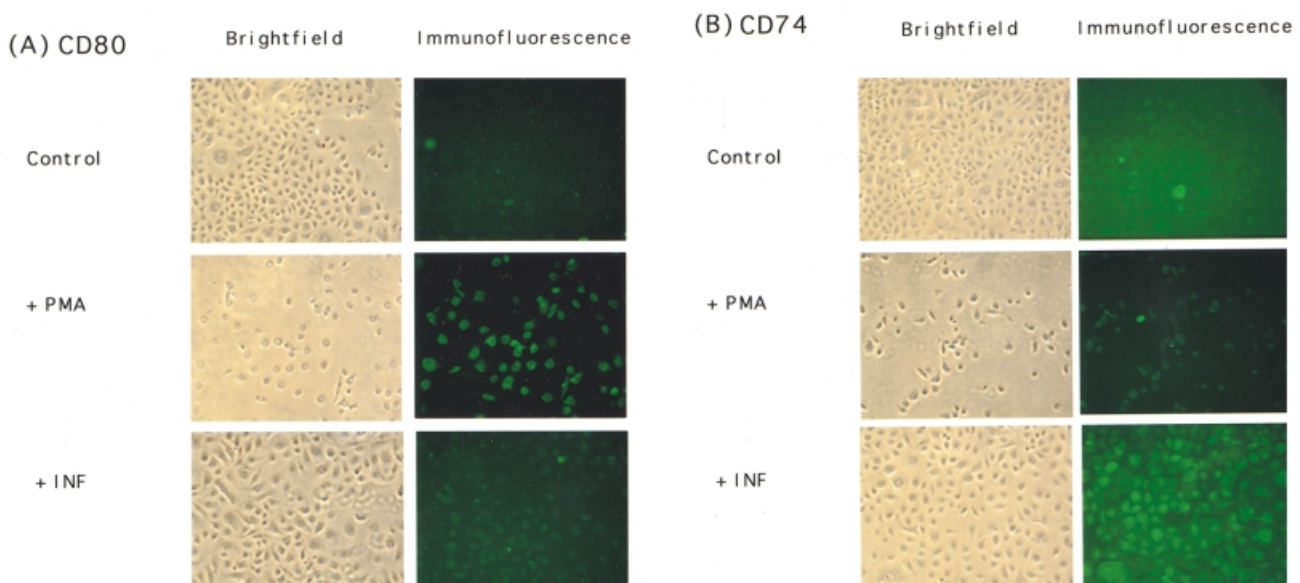

Figure 3. Cell surface expression of CD80 and CD74 in KC. KC were incubated in medium alone, or treated with either PMA (25 ng per ml) or IFN-γ (500 U per ml) for 48 h, then stained for expression of (*A*) CD80 (BB1 plus FITC-secondary antibody) or (*B*) CD74 (anti-CD74 plus FITC secondary antibody). Left-hand panels show bright field microscopy, whereas right panels show immunofluorescence microscopy. (*A*, *B*) The top panels are cells incubated in medium alone, middle panels are cells incubated with PMA and bottom panels are cells treated with IFN-γ (20× magnification for all panels).

fragment activity, and cells that do not express cell surface CD80 (fibroblasts) do not demonstrate CD80 promoter fragment activity. This suggests that CD80 promoter activity is cell specific and not promiscuous.

CD80 promoter mapping in human KC We transiently transfected our five different CD80 promoter constructs into human KC and determined the transcriptional activity of each construct (**Fig 5**). The three shortest promoter constructs showed activities which were significantly higher than the pGL3 vector alone in KC, with the highest activity occurring with pB7/514 (– 231), whereas pB7/367 and pB7/750 had much lower levels of activity. The longest constructs pB7/1132 and pB7/3709 had activities that were not significantly higher than the vector alone, suggesting that these longer fragments may contain transcriptional suppressor elements. Similar patterns of transcriptional activity were seen in Raji cells (a CD80+ B lymphocyte lineage cell line) by two other groups in which their shorter promoter constructs were more

active than longer constructs. Unlike Raji cells, KC did not require the SV40 enhancer to be present for activity.

PB7/514 promoter construct activity is upregulated in KC treated with allergens Human KC transfected with pB7/514 express a high basal level of luciferase (**Fig 5**). To determine whether chemicals implicated in ACD can induce enhanced CD80 promoter activity, we studied three different allergens: oxazolone, a chemical widely used in experimental ACD (Toews *et al*, 1980; Wilmer *et al*, 1994); Balsam of Peru, an aromatic liquid derived from the tree *Myroxolon balsumum* var. *perinae*, which is a common cause of skin allergy when incorporated into personal care products (Rudner *et al*, 1973); and nickel, a metal often found in jewelry, that is one of the most common causes of ACD in the world (Peltonen, 1979; Holness *et al*, 1995). All three allergens caused a concentration-dependent increase in CD80 gene reporter activity (**Fig 6a–c**). In representative experiments, KC treated with 0.2, 1.0, and 5.0 µg per ml oxazolone showed a 1.9-, 2.9-, and 11.2-fold

Table I. CSLC analysis of CD80 and CD74 expression by cultured KC

Conditions[a]	Ave. fluorescence (RFU)[b]	Ave. background (RFU)
CD80 analysis		
Medium alone	186	25
PMA treated	848	22
IFN-γ treated	262	22
CD74 analysis		
Medium alone	142	3
PMA treated	138	19
IFN-γ treated	414	3

[a]Culture conditions Medium alone-no added stimuli; PMA-treated-25 ng per ml for 48 h;

  IFN-γ-treated-500 U per ml for 48 h before staining.

[b]Relative fluorescence units.

Figure 4. Cell-specific expression of two CD80 promoter constructs in transfected Raji and human fibroblasts. Raji and fibroblast cells were transiently transfected with pB7e/367, pB7e/514, pB7e/750, pB7e/1132, pB73/3709, and pGL3e (vector alone) or pRL-CMV (positive transfection control) and incubated for 48 h. Cells were lyzed and the luciferase expression measured as rlu per μg extract protein and reported as relative expression compared with expression by cells transfected with the pGL3e vector alone. A schematic diagram of each of the CD80 promoter constructs used is shown to the left of the graph. Error bars represent the SEM value (n = 4). Key for figure: transfected human fibroblast cells, transfected Raji cells, pGL3 luciferase reporter vector, CD80 DNA.

increase in luciferase expression, respectively, compared with expression in untreated KC (compared with control, $p < 0.01, < 0.006$, and 0.005, respectively, highly statistically significant). KC treated with 20, 100, and 500 μg per ml Balsam of Peru had 1.5-, 2.5-, and 3.4-fold increase in luciferase expression (compared with control, $p < 0.006$, $p < 0.001$, and $p < 0.002$, respectively, highly statistically significant). Similar results were seen for the allergen $NiCl_2$ at 0.1 and 1.0 mM doses (**Fig 6c**) (compared with control, $p < 0.03$ and $p < 0.003$, respectively, highly statistically significant). Basal and inducible levels of luciferase expression, however, are reproducible in duplicate experiments using the same KC preparations. Basal levels of expression vary among KC prepared from different donors.

The pB7/514 promoter construct is transcriptionally upregulated in KC treated with irritants To determine whether the upregulation of CD80 was specific for allergens, we asked the question whether cutaneous irritants also caused an increase in CD80 pB7/514 promoter construct activity. Representative chemicals from this class of chemical contactants were as follows: dimethyl sulfoxide, a well known strong irritant and phenol, another potent irritant (Frosch, 1992). In a manner similar to allergen treatment, KC transfected with pB7/514 expressed higher levels of luciferase after treatment with either dimethyl sulfoxide or phenol (**Fig 7**). Treatment of transfected KC with 0.44, 2.2, and 11.0 μg per ml dimethyl sulfoxide increased luciferase expression by 2.5-, 3.2-, and 4.7-fold, respectively, compared with the basal level of luciferase expressed in untreated transfected KC (**Fig 7a**) (compared with control KC, $p < 0.01$, $p < 0.02$, and $p < 0.0005$, respectively, highly statistically significant). Treatment of KC with 6.6, 10, and 33 μg per ml phenol increased luciferase expression by 1.5-, 1.6-, and 2.0-fold, respectively, compared with the untreated control KC (**Fig 7b**) (compared with control KC $p < 0.02$, $p < 0.01$, and $p < 0.03$, respectively, highly statistically significant). Increased luciferase expression also occurred in KC treated with sodium lauryl sulfate (data not shown). Studies of inducible CD80 promoter expression with the other promoter fragments (see **Fig 1**) indicated that only the pB7/514 fragment was inducible in KC by treatment with allergens and irritants (data not shown).

DISCUSSION

There has been controversy as to the importance of CD80 costimulatory molecule gene expression in human KC, in normal skin, and in relation to the severity of inflammation seen during ACD and irritant contact dermatitis. The apparent enhanced expression of CD80 by KC *in situ* during inflammatory skin diseases (Nickoloff *et al*, 1993; Simon *et al*, 1994) seems to contradict their
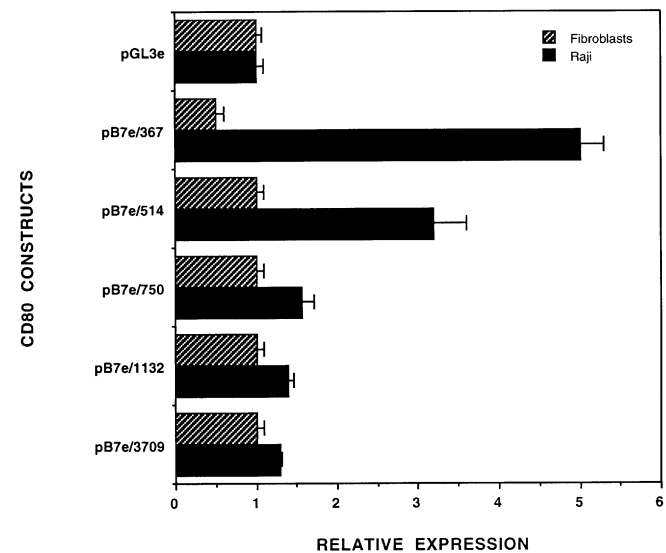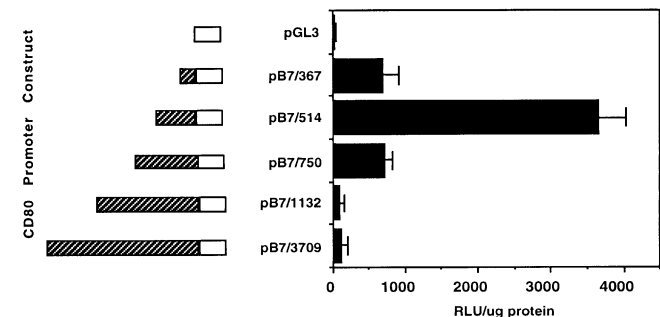

Figure 5. CD80 promoter mapping in transfected human KC. KC were transiently transfected with one of the five CD80 promoter constructs or the pGL3 vector alone. Transfected cells were incubated for 48 h then lyzed and the luciferase levels reported as rlu per μg cell extract protein. A schematic diagram of each of the CD80 promoters used is shown to the left of the graph. The error bars are the SEM value (n = 4). Key for figure: pGL3 luciferase reporter vector, CD80 DNA.

proposed role as tolerogenic APC (Gaspari *et al*, 1988; Bal *et al*, 1990; Gaspari and Katz, 1991). Other studies (Hagerty *et al*, 1994) suggest that it is actually the expression of costimulatory molecules by certain tissues and not class II MHC antigen expression that determines the extent of a $CD4^+$ T lymphocyte-mediated inflammatory response in a diseased tissue. This paradigm implies that the induced CD80 expression by epidermal KC in some dermatologic diseases may also be a contributing factor for the magnitude of inflammation in the skin.

We have shown that cultured human KC can express CD80 on their cell surfaces as measured by staining with the MoAb, BB-1. Nickoloff *et al*, 1993) also have reported that KC show a low basal level of CD80 expression when stained with BB-1, which was increased when KC are activated by treatment with IFN-γ plus 12-O-tetradecanoyl phorbol 13-acetate ester. Other anti-CD80

MoAb, however, did not react with these activated KC (Nickoloff *et al*, 1993). Two theories have been advanced to deal with this discrepancy. The first theory suggests that BB-1 binds to CD80, as well as, a third B7 molecule, B7-3, which would encode a separate, but related gene (Boussiotis *et al*, 1993). No direct evidence for the

existence of B7-3 has been reported so far. The second theory suggests that BB-1 staining may be the result of both CD74 expression, as well as, CD80 expression. Recently, BB-1 has been shown to bind to both cell surface CD80 and CD74 (MHC class II associated invariant chain) on pre-B acute lymphocytic leukemia cells (Freeman *et al*, 1998). To address this question, we analyzed the cell surface expression of both BB-1 and CD74 in untreated
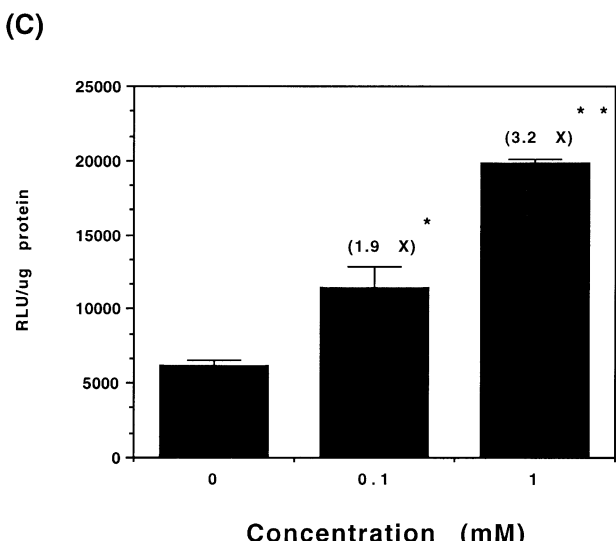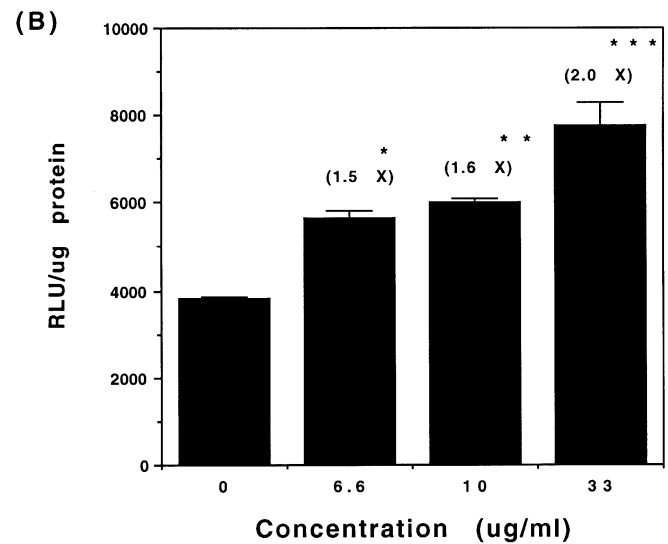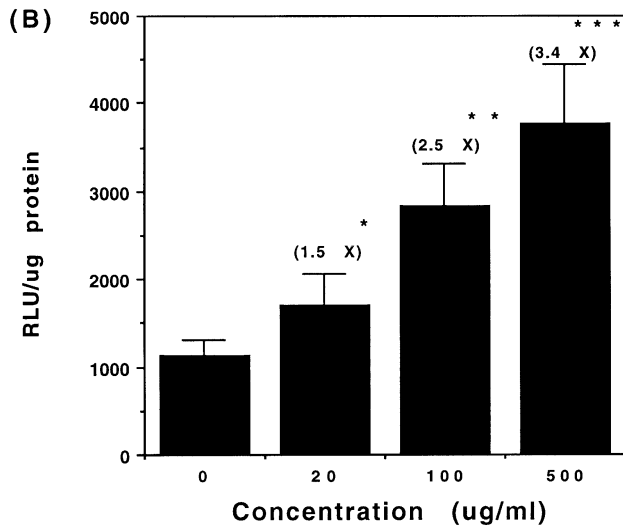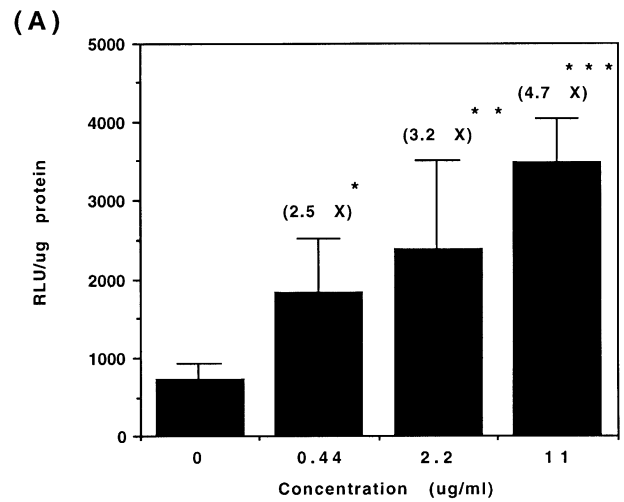
(A)

(B)
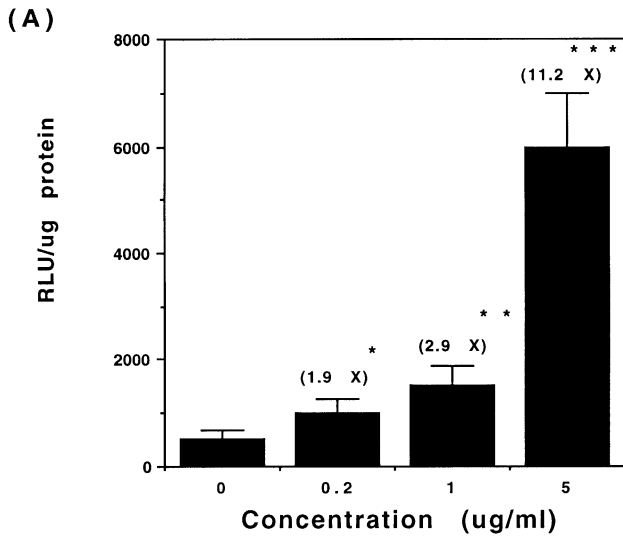

(C)

(A)

(B)

Figure 7. Activity of the pB7/514 CD80 promoter construct is upregulated in transfected human KC treated with irritants. Conditions were the same as described in the legend to **Fig 7**, except cells were treated with the irritants (*A*) dimethyl sulfoxide (compared with control: *$p < 0.014$, **$p < 0.022$, ***$p < 0.005$) or (*B*) phenol (compared with control: *$p < 0.025$, **$p < 0.013$, ***$p < 0.032$).

Figure 6. Activity of the pB7/514 CD80 promoter construct is upregulated in transfected human KC treated with allergens. KC were transiently transfected with the pB7/514 CD80 promoter construct and incubated for 16 h, followed by treatment with an allergen, either (*A*) oxazolone (compared with control: *$p < 0.014$, **$p < 0.006$, ***$p < 0.005$ significant) or (*B*) Balsam of Peru (compared with control: *$p < 0.0063$, **$p < 0.0013$, ***$p < 0.0027$) or (*C*) NiCl$_2$ (compared with control *$p < 0.038$, **$p < 0.0031$) for 48 h post-transfection. Cells were lyzed and luciferase levels reported as rlu per µg cell extract protein vs. the concentration of allergen used. The increase in rlu per µg protein in treated cells compared with the untreated cells is denoted above each bar on the graph. Error bars are the SEM value (n = 4).

KC and KC treated with either IFN-γ or PMA. As shown in **Fig 3**, CD74 is not expressed in untreated or PMA-treated KC but is induced in IFN-γ-treated KC, whereas BB-1 is only increased after PMA treatment and not after IFN-γ treatment. The different pattern of cell surface expression for BB-1 compared with CD74 in KC under these conditions, indicates that BB-1 is not staining CD74 in KC, and BB-1 reactivity may represent CD80 expression by KC.

Furthermore, CD80 promoter studies show that the CD80 gene is transcriptionally active in KC and this activity is upregulated in KC treated with allergens and irritants. The discrepancy in staining between BB-1 and other CD80 MoAb in KC may be the result of alternate splicing of the CD80 mRNA, producing a CD80 epitope, which is only recognized by BB-1 and not other CD80 MoAb. Further evidence to support this theory includes CD80 RNA transcript analysis which shows that whereas B cells (which react with all anti-CD80 reagents), express a full array of 1.7 kb, 2.4 kb, 4.2 kb, and 10 kb transcripts, cultured KC (which react only with the BB-1 MoAb) express only the 1.7 kb and 2.4 kb transcripts, but not the 4.2 or 10 kb transcripts (Nasir *et al*, 1995).

Previous studies of a CD80 enhancer element suggested that this regulatory element was active in cells of the B cell lineage, but not in cells derived from epithelial cell lineage (Zhao *et al*, 1996). These investigators, however, focused their studies on a transformed, long-term epithelial cell line (HeLa), but did not examine cultured, normal human KC. Our studies of normal human KC are the first to demonstrate that CD80 regulatory elements are constitutively active in this cell type, and are inducible by exposure to chemical allergens and irritants. Our studies of basal and inducible CD80 promoter fragment expression (pB7/514) by KC is very similar to the results of promoter fragment mapping by professional APC (Zhao *et al*, 1996), in which these smaller, proximal fragments of the CD80 promoter exhibited the highest levels of constitutive and inducible activity. Thus, this pattern of CD80 promoter fragment activity is likely to be of physiologic significance in professional APC and KC.

A possible mechanism for the perturbations of CD80 gene expression by this diverse array of chemical allergens and irritants may involve alterations in KC redox state. It is well known that alterations in cellular redox potential induce a number of different transcription factors that control gene activation by DNA binding and transactivation of many genes encoding immunologically relevant molecules (Abate *et al*, 1990; Xanthoudakis *et al*, 1992; Meyer *et al*, 1993). Ongoing experiments are addressing this possibility.

The observations that both CD80 transcriptional activity and cell surface expression were upregulated in KC treated with the allergens, $NiCl_2$, oxazolone and Balsam of Peru, and the irritants, sodium lauryl sulfate, dimethyl sulfoxide, and phenol, indicate the importance of the CD80 costimulatory molecule in the breaking of immunologic tolerance of the skin in cases of ACD and irritant contact dermatitis. Additional evidence of this importance is demonstrated in studies of transgenic mice that overexpress the CD80 gene in their KC. These transgenic mice exhibit an exaggerated and persistent cutaneous delayed-type hypersensitivity when exposed to either allergens or irritants compared with nontransgenic mice (Nasir *et al*, 1994 Gaspari *et al*, 1998). In comparison, transgenic mice which overexpress CD86 at a level similar to the CD80 transgenic mice, developed a persistent delayed-type hypersensitivity, which was intermediate in severity between nontransgenic and CD80 transgenic mice.[1] This suggests that, in KC, CD80 gene expression is more important than CD86 expression in amplifying T lymphocyte-mediated inflammation during contact dermatitis.

All these observations lend support to Matzinger's "danger signal" hypothesis (Matzinger, 1998), which suggests that immune responses are initiated when cells are destroyed or stressed, These cells release or express danger signals for the immune system. Costimulatory molecules such as CD80 may be one of those signals, as costimulation is required for immune activation of T helper cells. KC would be an important component in this process because of their ability to express CD80 after induction by harmful chemicals. In normal skin, KC express very little CD80 and play a part in maintaining peripheral tolerance in the skin due to their defective antigen presentation to T cells. In contrast, KC that have been chemically stressed, as in the case of percutaneous exposure to allergens or irritants, greatly increase their CD80 expression. This may allow the breakdown of immunologic tolerance in the skin, resulting in T helper cell-mediated inflammation for both ACD and irritant contact dermatitis.

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[1]Burns RP, Nasir A, Ferbel B, *et al*: The T cell costimulatory molecules B7-1 (CD80) and B7-2 (CD86) when expressed on keratinocytes deliver different signals during contact hypersensitivity responses. *J Invest Dermatol* 110:477, 1998 (abstr.)

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