Regulation of Adrenomedullin Secretion in Cultured Human Skin and Oral Keratinocytes

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Adrenomedullin, a potent vasoactive peptide, is actively secreted from primary cultures of human oral and skin keratinocytes, but nothing is known of the regulation of its release. This study describes the effects of a range of substances on adrenomedullin production from cultures of oral and skin keratinocytes. We have established that keratinocytes do not store adrenomedullin but secrete it constitutively. Cytokines interleukin-1α and -1β, tumor necrosis factor-α and -β, and the bacterial product, lipopolysaccharide, significantly stimulate adrenomedullin secretion from oral but not skin keratinocytes. Both transforming growth factor-β1 and interferon-γ are potent suppressors of adrenomedullin secretion from both cell types, as are forskolin, di-butyryl cyclic adenosine monophosphate, and adrenocorticotropin. The peptides thrombin and endothelin-1 increase adrenomedullin production, particularly from skin keratinocytes. These findings indicate that there are differences in the regulation of adrenomedullin production between oral and skin keratinocytes and that oral keratinocytes are particularly responsive to the action of inflammatory cytokines. This raises the possibility that adrenomedullin may serve a different functions in oral mucosa and skin. ***Key words: cytokines/peptide regulation/peptides secretion/primary cell culture. J Invest Dermatol 117:353–359, 2001***

Keratinocytes form the epithelial barrier in oral mucosa and skin and as well as maintaining structural integrity play an active part in local immune defense. They synthesize and release inflammatory cytokines, and chemokines such as interleukin (IL) -1, IL-6, tumor necrosis factor (TNF) -α, IL-12, IL-8, and RANTES in response to a variety of environmental stimuli, including the bacterial product, lipopolysaccharide (LPS), ultraviolet light, and other inflammatory cytokines (reviewed in Kondo, 1999). Production of these cytokines modulates the activities of other keratinocytes, fibroblasts, endothelial cells, and leukocytes, and is important in the generation of local immune and inflammatory responses.

In addition to inflammatory cytokines, keratinocytes produce a variety of growth factors and peptides. One of these is adrenomedullin (AM), a potent vasoactive peptide first extracted from a human pheochromocytoma (Kitamura *et al*, 1993a). Human AM is a 52 amino acid peptide with a single disulfide bridge between residues 16 and 21 and with an amidated tyrosine at the carboxy terminus (Kitamura *et al*, 1993b). It shows modest structural homology with calcitonin gene-related peptide and is a member of the calcitonin gene-related peptide/amylin peptide family. The AM gene is expressed in a wide range of tissues (reviewed in Hinson *et al*, 2000 and references therein). An initial report on the distribution of AM mRNA suggested that the highest levels of expression were seen in the adrenal medulla, ventricle, kidney, and lung. Since the discovery that the AM gene is even more highly expressed in endothelial cells than in the adrenal medulla, this peptide together with nitric oxide and endothelin (ET), have come to be regarded as secretory products of the vascular endothelium (Hinson *et al*, 2000).

Specific AM receptors have been identified in a variety of studies, using radioligand-binding techniques, and these receptors are coupled to adenylyl cyclase, thus elevating cAMP levels when activated (Eguchi *et al*, 1994; Ishizaka *et al*, 1994). Specific receptors for AM are also present on oral and skin keratinocytes (Kapas *et al*, 1997; Martínez *et al*, 1997) and there is evidence these are functional: AM stimulates keratinocyte proliferation and this effect may be mimicked by cAMP (Kapas *et al*, 1997).

Although it has been established that skin keratinocytes synthesize AM (Martínez *et al*, 1997) it is not known whether it is also produced by oral keratinocytes. In addition nothing is known of the factors that influence its production and hence of its physiologic role in the skin and oral mucosa. The aims of this study were to determine whether oral keratinocytes produce AM and to establish the factors important in modulating its release from oral and skin keratinocytes.

MATERIALS AND METHODS

Materials Dexamethasone, aldosterone, hydrocortisone, testosterone, progesterone, estradiol, LPS, bovine thrombin, forskolin, di-butyryl cAMP, and 12-*O*-tetradecanoylphorbol13-acetate (TPA) were purchased from Sigma-Aldrich (Poole, U.K.). Human recombinant IL-1α, IL-1β, TNF-α, TNF-β, transforming growth factor (TGF)-β1, and interferon (IFN)-γ were purchased from R&D Systems (Abingdon, U.K.). ET-1 and adrenocorticotropin (ACTH) (Synacthen) were obtained from Bachem (Saffron Walden, U.K.) and Ciba-Geigy (Horsham, Sussex,

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Abbreviations: ACTH, adrenocorticopin; AM, adrenomedullin; ET, endothelin-1.

Table I. AM production from keratinocytes increased with time[a]

Time point	Secreted AM (fmol per 10^6 cells)		Intracellular AM (fmol per 10^6 cells) (h)	
	Oral keratinocytes	Skin keratinocytes	Oral keratinocytes	Skin keratinocytes
0	7.4 ± 0.05	10.1 ± 1.6	0.27 ± 0.01	0.31 ± 0.04
1	10.2 ± 0.9*	15.6 ± 1.2*	0.33 ± 0.05	0.37 ± 0.09
2	15.3 ± 1.3*	21.3 ± 1.8*	0.31 ± 0.03	0.35 ± 0.05
6	21.9 ± 1.9*	25.8 ± 2.3*	0.29 ± 0.03	0.34 ± 0.04
12	25.4 ± 2.9*	35.2 ± 2.8*	0.28 ± 0.05	0.38 ± 0.05

[a]Rate of intracellular and secreted AM levels from oral and skin keratinocytes. Each value represents mean ± SEM, n = 4. *$p < 0.05$ compared with levels at time 0 h (one-way ANOVA followed by a Dunnett's test).

U.K.), respectively. Tissue culture media, reagents, and plastics were obtained from Life Technologies (Paisley, Scotland). AM enzyme immunoassay kits and human AM(1–52) peptide were purchased from Phoenix Pharmaceuticals (Belmont, CA).

Cell culture Primary human oral and skin keratinocytes were derived from biopsy material that was determined to be normal by histologic methods as described in detail elsewhere (Rheinwald and Green, 1975). Cells were grown in 75 cm^2 tissue culture flasks on a feeder layer of γ-irradiated Swiss 3T3 fibroblasts as described elsewhere (Malcovati and Tenchini, 1991; Li *et al*, 2000). Cells were maintained in keratinocyte growth medium supplemented with 10% fetal bovine serum and antibiotics in an humidified atmosphere containing 5% CO_2 and 95% air at 37°C as described previously (Kapas *et al*, 1998a). Twenty-four hours prior to an experiment cells (from passages 3 to 5) were detached from the flasks and seeded on to 24-well plates or in 75 cm^2 tissue culture flasks and maintained, without a feeder layer, in serum-free keratinocyte growth medium.

Measurement of AM Human keratinocytes, grown to 80% confluence in 24-well tissue culture plates, were washed twice in sterile phosphate-buffered saline and incubated in serum-free keratinocyte growth medium in the absence and presence of various agents for different lengths of time. After the incubation period, conditioned medium was harvested, centrifuged and the supernatant stored at –20°C until assayed. The cells were washed in sterile phosphate-buffered saline, scraped in 1 M acetic acid, sonicated, and centrifuged, and the resulting supernatants were subjected to enzyme immunoassay tests for AM following the manufacturer's instructions (Phoenix Pharmaceuticals). The lower detection limit of the assay was 12 fmol AM per assay; interassay and intra-assay coefficients of variance were 14 and 8%, respectively, at 40 fmol AM per tube (n = 20).

RNA analysis Total cellular RNA, reverse transcription, and polymerase chain reaction (PCR) was performed as described previously (Kapas *et al*, 1998a). The sequences of the oligonucleotide primers were as follows: AM sense: 5′-atgaagctggtttccgtc-3′ and anti-sense 5′-tgtggcttagaagacacc-3′, and GAPDH sense: 5′-ccacagtccatgccatcac-3′ and anti-sense: 5′-tccaccaccctgttgctgta-3′. PCR products were electrophoresed through 1% agarose gels and viewed by ultraviolet illumination and photographed. The PCR bands underwent scanning densitometry and the relative ratio of the net intensities of the AM and GAPDH bands from the same PCR reaction was determined to show AM mRNA expression in response to exposure to various agents.

All experiments were carried in duplicate on four separate occasions and bar graphs represent the results obtained by pooling data together. The reverse transcription–PCR data are representative of each experiment.

Statistical analysis Arithmetic means and standard error of the means were calculated. One-way analysis of variance was used to test whether factors had an effect on basal (control) levels of cAMP, and Dunnett's test was used to determine whether agents affected stimulated events using Minitab statistics software package (Daniel, 1976).

RESULTS

AM secretion from keratinocytes change with time The cellular content of AM and its accumulation in the culture medium of human oral and skin keratinocytes (keratinocytes) was measured after incubation periods of 1, 2, 6, and 12 h. **Table I** shows the AM content in the culture medium increased throughout the period of measurement with skin keratinocytes secreting 40% more AM than oral keratinocytes. The rate of secretion of AM from the cultures of oral and skin keratinocytes was 2.08 fmol per 10^6 cells per h and 2.91 fmol per 10^6 cells per h as an average over a 12 h period, respectively. In contrast, apart from an increase in cellular AM levels after 1 h in both oral and skin keratinocytes (**Table I**), the cellular content of AM remained unchanged, suggesting AM was not stored but was secreted constitutively.

Table II. Effects of cytokines and steroids on AM secretion[a]

Substance	Concentration	Oral keratinocytes (fmol AM per 10^6 cells)	Skin keratinocytes (fmol AM per 10^6 cells)
Basal	–	25.4 ± 2.9	35.2 ± 2.8
IL-1α	0.01 ng per ml	30.5 ± 2.8*	34.6 ± 3.1
IL-1β	0.1 ng per ml	31.5 ± 2.9*	35.2 ± 3.1
TNF-α	1 ng per ml	45.6 ± 3.6*	32.8 ± 3.0
TNF-β	10 ng per ml	42.9 ± 4.0*	36.9 ± 4.2
LPS	10 ng per ml	50.2 ± 4.5*	36.8 ± 4.6
TGF-β1	1 ng per ml	20.9 ± 3.2*	25.3 ± 3.1*
IFN-γ	100 U per ml	21.3 ± 3.1*	28.3 ± 3.1*
Thrombin	20 U per ml	29.6 ± 1.7*	56.3 ± 4.1*
Hydrocortisone	10^{-5} mol per liter	29.8 ± 2.5	39.4 ± 2.1*
Aldosterone	10^{-5} mol per liter	28.5 ± 1.1	36.2 ± 1.2*
Dexamethasone	10^{-5} mol per liter	28.6 ± 1.6	37.7 ± 0.28*
Testosterone	10^{-5} mol per liter	29.9 ± 3.0	38.2 ± 2.1*
Progesterone	10^{-5} mol per liter	28.3 ± 1.6	39.1 ± 2.8*
Estradiol	10^{-5} mol per liter	29.9 ± 2.5	38.6 ± 2.9*
TPA	10^{-9} mol per liter	35.5 ± 3.4*	51.8 ± 1.5*
ET-1	10^{-9} mol per liter	38.8 ± 3.9*	49.3 ± 4.1*
Forskolin	10^{-6} mol per liter	19.3 ± 1.5*	30.2 ± 3.1*
Di-butyryl cAMP	10^{-6} mol per liter	22.6 ± 2.1*	32.2 ± 3.5
ACTH	10^{-9} mol per liter	19.3 ± 2.8*	22.8 ± 2.5*

[a]Human oral and skin keratinocytes were cultured with various substances for 12 h. Each value represents mean ± SEM, n = 4. *$p < 0.05$ compared with basal (control) levels (one-way ANOVA followed by a Dunnett's test).

AM secretion from keratinocytes is regulated by various agents Based on data of regulation of AM secretion from cultured vascular smooth muscle and endothelial cells (Isumi *et al*, 1998), cells were exposed to a variety of agents to test their effects on AM secretion after a 12 h incubation period. **Table II** shows that most substances tested significantly influenced AM secretion from oral and skin keratinocytes. For oral keratinocytes eight agents increased and five substances decreased AM secretion, whereas for skin keratinocytes, nine substances increased, four substances decreased and six had no effect on AM secretion. Cytokines IL-1α, IL-1β, TNF-α and TNF-β all increased AM secretion

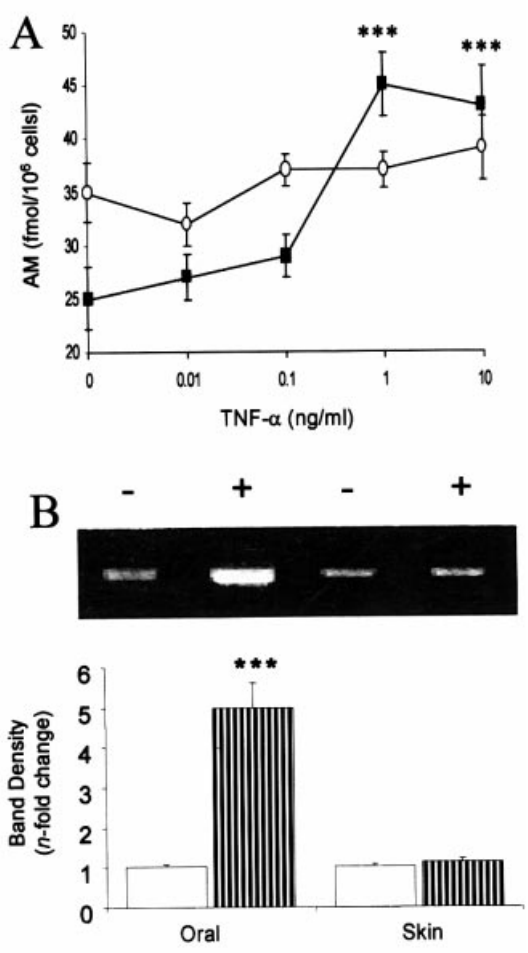

Figure 1 TNF-α caused significant increases in AM protein and mRNA levels from keratinocytes. Effects of increasing concentrations of TNF-α on (*A*) AM levels in the culture medium of human oral (■) or skin keratinocytes (○) after incubation for 12 h; (*B*) AM mRNA expression in keratinocytes before (–) and after (+) 12 h exposure to TNF-α (1 ng per ml); upper picture is a representative image of AM mRNA levels; lower graph shows relative intensity of AM mRNA expression after scanning densitometry and normalization to GAPDH expression. Each value represents mean ± SEM, n = 4. ***p < 0.001 compared with control (no treatment) levels (one-way ANOVA followed by a Dunnett's test).

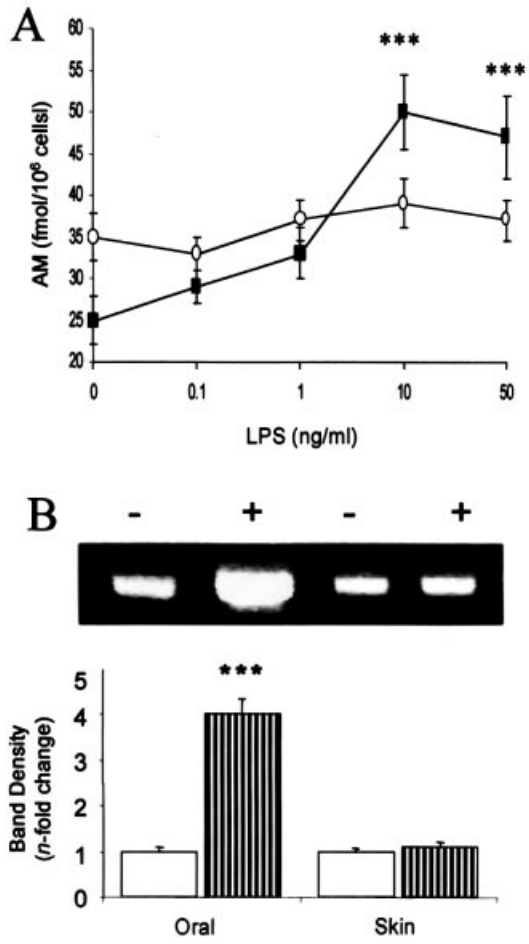

Figure 2. LPS caused significant increases in AM protein and mRNA levels from keratinocytes. Effects of increasing concentrations of LPS on (*A*) AM levels in the culture medium of human oral (■) or skin keratinocytes (○) after incubation for 12 h; (*B*) AM mRNA expression in keratinocytes before (–) and after (+) 12 h exposure to LPS (10 ng per ml); upper picture is a representative image of AM mRNA levels; lower graph shows relative intensity of AM mRNA expression after scanning densitometry and normalization to GAPDH expression. Each value represents mean ± SEM, n = 4. ***p < 0.001 compared with control (no treatment) levels (one-way ANOVA followed by a Dunnett's test).

significantly from oral keratinocytes but had no effect on skin keratinocytes. The greatest increase was seen with TNF-α and TNF-β, which almost doubled secretion over the 12 h period. As shown in **Fig 1**(***A***), significant increases in AM secretion from oral keratinocytes were not observed until 1 ng TNF-α per ml was used. No change in AM secretion from skin keratinocytes was seen at any concentration. This was also reflected at the mRNA level as depicted in **Fig 1**(***B***). A similar dichotomy of response between oral and skin keratinocytes was seen with LPS: AM secretion more than doubled from oral keratinocytes but skin keratinocytes did not respond. Threshold stimulation of oral keratinocytes with LPS occurred at 10 ng per ml (**Fig 2***A*). **Figure 2**(***B***) illustrates the effect of a maximal amount of LPS (10 ng per ml) on AM mRNA level in both oral and skin cells.

TPA caused concentration-dependent increases in AM secretion from both cell types (between 10^{-9} and 10^{-8} mol per liter), but at higher concentrations, TPA did not have a stimulatory effect and AM levels returned to basal levels (**Fig 3***A*). **Figure 3**(***B***) shows that 10^{-9} mol TPA per liter caused an increase in AM expression, more so in skin keratinocytes than oral.

In both cell types TGF-β1 and IFN-γ were potent suppressors of AM secretion decreasing production by up to third. These effects were concentration-dependent. **Figure 4**(***A***) illustrates the attenuation of AM secretion from both oral and skin keratinocytes by TGF-β1; threshold inhibition of production occurred at 1 ng TGF-β1 per ml for both cell types. The effect of this concentration of TGF-β1 on AM mRNA expression can be seen in **Fig 4**(***B***).

Thrombin stimulated AM secretion from both oral and skin keratinocytes. Skin keratinocytes, however, were more responsive than oral keratinocytes and secretion almost doubled compared with only a 15% increase from oral keratinocytes (**Table II**). ET-1 also stimulated AM secretion significantly from both oral and skin keratinocytes (**Table II**) and **Fig 5**(***A***) illustrates the concentration-dependent increase. The effects of 10^{-9} mol ET-1 per liter on AM gene expression are shown in **Fig 5**(***B***).

The effect of six steroid and sex hormones on AM secretion from keratinocytes were studied. **Table II** illustrates that skin keratinocytes responded to all steroids used and increased AM production by about 10% when compared with control levels. There was little difference in the ability of the hormones to stimulate AM secretion.

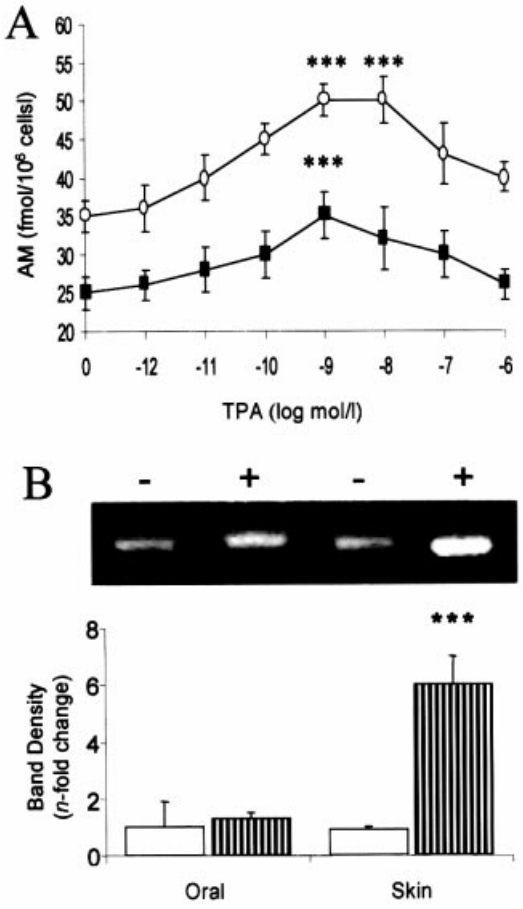

Figure 3. TPA caused a significant increase in AM protein and mRNA levels from keratinocytes. Effects of increasing concentrations of TPA on (*A*) AM levels in the culture medium of human oral (■) or skin keratinocytes (○) after incubation for 12 h; (*B*) AM mRNA expression in keratinocytes before (–) and after (+) 12 h exposure to TPA (10^{-9} mol per liter); upper picture is a representative image of AM mRNA levels; lower graph shows relative intensity of AM mRNA expression after scanning densitometry and normalization to GAPDH expression. Each value represents mean ± SEM, n = 4. ***p < 0.001 compared with control (no treatment) levels (one-way ANOVA followed by a Dunnett's test).

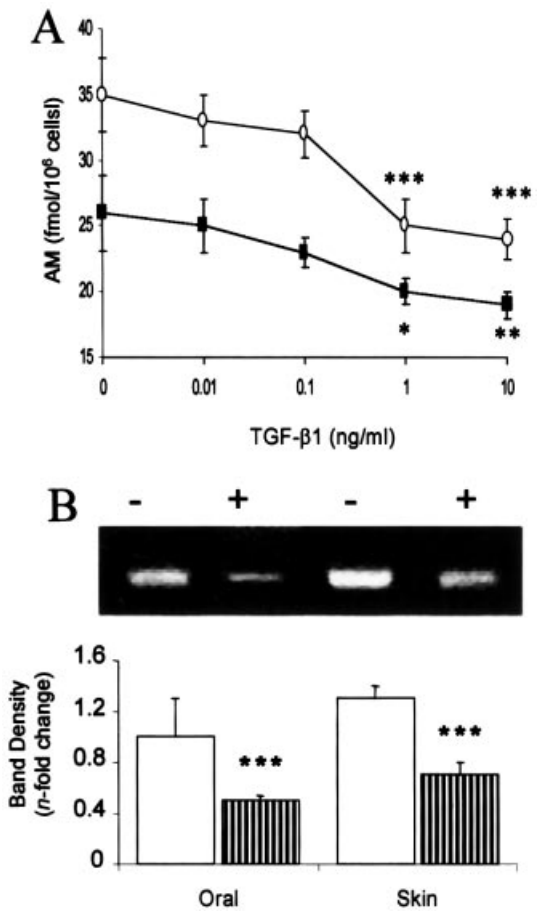

Figure 4. TGF-β1 caused significant attenuation of AM protein and mRNA levels from keratinocytes. Effects of increasing concentrations of TGF-β1 on (*A*) AM levels in the culture medium of human oral (■) or skin keratinocytes (○) after incubation for 12 h; (*B*) AM mRNA expression in keratinocytes before (–) and after (+) 12 h exposure to TGF-β1 (1 ng per ml); upper picture is a representative image of AM mRNA levels; lower graph shows relative intensity of AM mRNA expression after scanning densitometry and normalization to GAPDH expression. Each value represents mean ± SEM, n = 4. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control (no treatment) levels (one-way ANOVA followed by a Dunnett's test).

Thyroid hormone, T_3, did not affect AM secretion (data not shown). Oral keratinocytes appeared not to respond to steroid hormones.

ACTH, forskolin and di-butyryl cAMP significantly attenuated AM secretion in both oral and skin keratinocytes (**Table I**). **Figure 6** illustrates the significant concentration-dependent inhibitory affect of ACTH on AM production and mRNA levels. Threshold inhibition occurred at 10^{-9} mol ACTH per liter for skin keratinocytes and 10^{-10} mol ACTH per liter for oral keratinocytes. At the gene level 10^{-8} mol ACTH per liter did attenuate expression, particularly in skin keratinocytes.

IL-1α modulates AM production in response to TNF-α or LPS TNF-α, IL-1α and LPS may act synergistically when added simultaneously (Isumi *et al*, 1998) and the effect of these substances in combination on AM secretion from oral keratinocytes was studied. Each of the three substances individually increased AM production (**Fig 7**), but only TNF-α and LPS in combination significantly increased secretion over levels seen with either substance alone. This effect was additive rather than synergistic. Interestingly, addition of IL-1α to a combination of TNF-α and LPS prevented this additive effect and levels of AM production were similar to TNF-α on its own. IL-1α also had an inhibitory effect when added in combination with LPS and levels were similar to that of IL-1α on its own.

DISCUSSION

Although skin keratinocytes have been shown previously to secrete AM, this is the first demonstration that oral keratinocytes produce the peptide. Primary cultures of both oral and skin keratinocytes were shown to synthesize and secrete AM constitutively. The rate of AM secretion from both keratinocytes types was comparable with that of rat vascular smooth muscle cells (Sugo *et al*, 1994a), but about six times less than vascular endothelial cells (Isumi *et al*, 1998). The level of gene transcription of AM in the cultured keratinocytes was lower than that of the rat adrenal gland and vascular endothelial cells suggesting that keratinocytes are not a major source of AM synthesis or secretion within the body.

In order to define a physiologic role for AM in oral mucosa and skin it is important to establish which factors affect the regulation of AM production by oral and skin keratinocytes. Interesting differences were found between the two cell types. The constitutive level of secretion of AM from skin keratinocytes was about 40% greater than from oral keratinocytes and, although the maximal level of secretion following stimulation was similar for

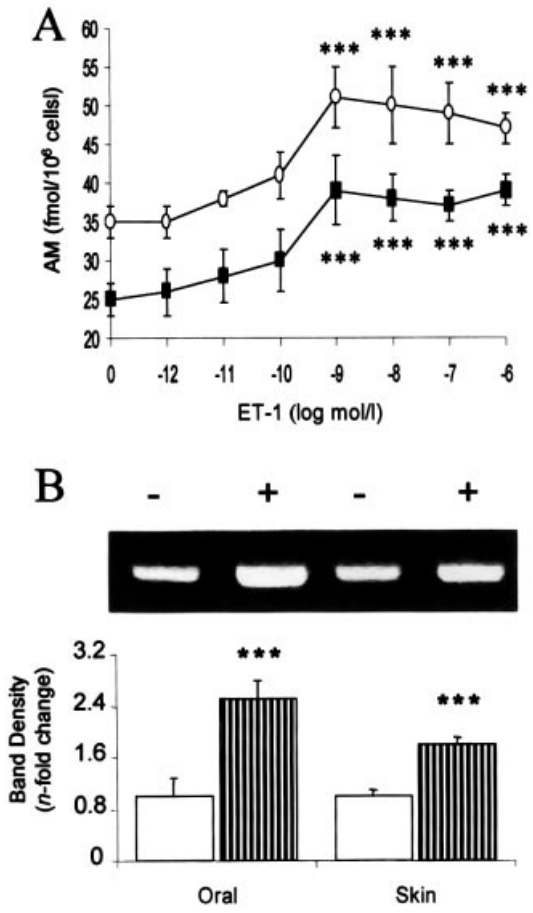

Figure 5. ET-1 caused significant increases in AM protein and mRNA levels from keratinocytes. Effects of increasing concentrations of ET-1 on (*A*) AM levels in the culture medium of human oral (■) or skin keratinocytes (○) after incubation for 12 h; (*B*) AM mRNA expression in keratinocytes before (–) and after (+) 12 h exposure to ET-1 (10^{-9} mol per liter); upper picture is a representative image of AM mRNA levels; lower graph shows relative intensity of AM mRNA expression after scanning densitometry and normalization to GAPDH expression. Each value represents mean ± SEM, n = 4. ***p < 0.001 compared with control (no treatment) levels (one-way ANOVA followed by a Dunnett's test).

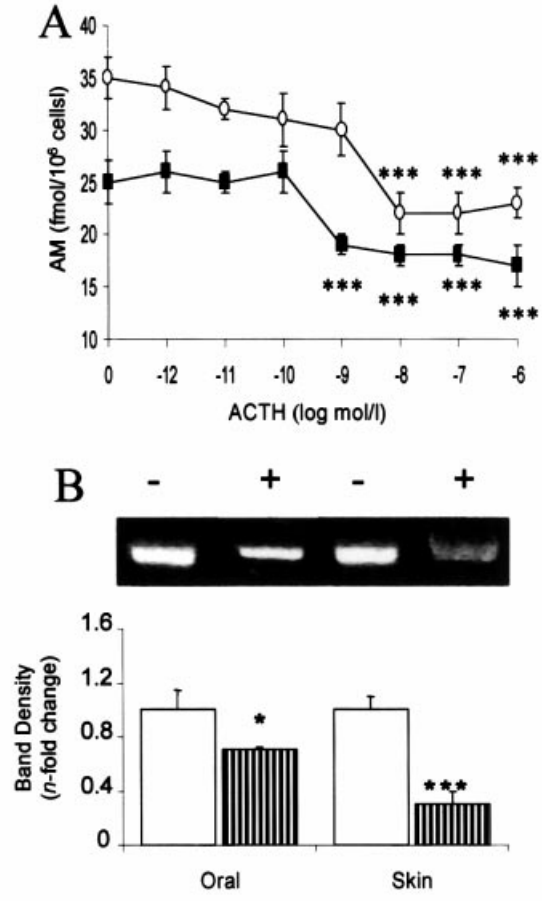

Figure 6. ACTH caused significant decreases in AM protein and mRNA levels from keratinocytes. Effects of increasing concentrations of ACTH on (*A*) AM levels in the culture medium of human oral (■) or skin keratinocytes (○) after incubation for 12 h; (*B*) AM mRNA expression in keratinocytes before (–) and after (+) 12 h exposure to ACTH (10^{-8} mol per liter); upper picture is a representative image of AM mRNA levels; lower graph shows relative intensity of AM mRNA expression after scanning densitometry and normalization to GAPDH expression. Each value represents mean ± SEM, n = 4. *p < 0.05, ***p < 0.001 compared with control (no treatment) levels (one-way ANOVA followed by a Dunnett's test).

both cell types, the keratinocytes responded differently to a number of the agents tested. Oral keratinocytes significantly increased output of AM in response to the pro-inflammatory cytokines IL-1α, IL-1β, TNF-α, and TNF-β, whereas skin keratinocytes did not respond. In addition, oral keratinocytes were particularly responsive to the bacterial product, LPS, and AM production doubled, whereas it had no effect on skin keratinocytes. In contrast, skin keratinocytes were highly responsive to thrombin but the effect on oral keratinocytes was much less marked. The significance of these differences in terms of the function of AM secretion by keratinocytes in oral mucosa and skin is not known. Nonetheless the finding that AM production from oral keratinocytes is stimulated by pro-inflammatory cytokines and bacterial products suggests AM may play an important part in immune defense of the oral mucosa, particularly in response to microorganisms. This is further suggested by our recent findings that AM induces intercellular adhesion molecule 1 (ICAM-1) and E-selectin expression on oral keratinocytes and endothelial cells *in vitro* as well as stimulating the release of the IL-1α and IL-6 (Farthing *et al*, 1999; Hagi-Pavli *et al*, in press). E-selectin together with ICAM-1 on vascular endothelium are important in mediating the migration of neutrophils (Lawrence and Springer, 1993) as well as a specific subset of leukocytes characterized by the expression of cutaneous associated lymphocyte antigen from peripheral blood into the oral mucosa and skin (Picker *et al*, 1991; Walton *et al*, 1997). IL-1α and IL-6 play a pivotal part in the immune response by stimulating the release of cytokines from a variety of cell types, including keratinocytes and endothelial cells and by activating T cells and Langerhans cells (Kondo, 1999; Murphy *et al*, 2000). AM itself may therefore upregulate the immune response in oral mucosa, particularly in response to bacterial products.

Combinations of cytokines have been shown to have a synergistic or additive effect on the production of cytokines by oral and skin keratinocytes (Li *et al*, 1996). The effect of a combination of IL-1α, TNF-α, and LPS on AM release from oral keratinocytes was examined, but only TNF-α and LPS together resulted in a significant additive increase in AM secretion. IL-1α in combination with both LPS and TNF-α or LPS on its own had an inhibitory effect on AM secretion. Similar observations have been reported in studies using primary cultures of rat endothelial cells (Isumi *et al*, 1998). It is not clear why IL-1 should have both a stimulatory and inhibitory effect on AM secretion but these results indicate the complexity of control of AM release that may occur *in vivo*.

TPA elicited two effects on AM production from both oral and skin keratinocytes: low concentrations increased AM secretion, but

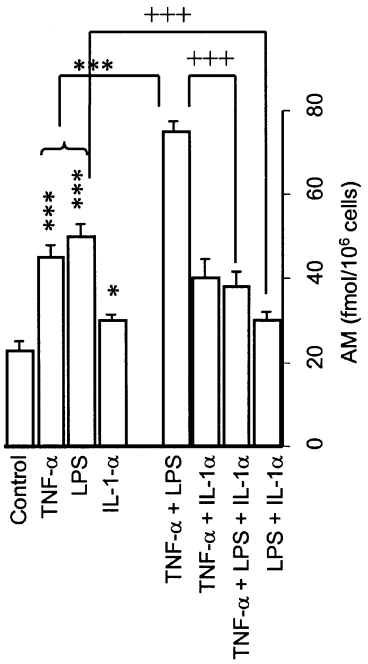

Figure 7 IL-1α attenuated LPS- or TNF-α-stimulated AM production from oral keratinocytes. Effects of coincubating TNF-α (1 ng per ml), LPS (10 ng per ml), and IL-1α (0.01 ng per ml) for 12 h on AM secretion from oral keratinocytes. Each value represents mean ± SEM, n = 4. *p < 0.05, ***p < 0.001 compared with control levels; +++p < 0.001 compared with AM levels stimulated by TNF-α, LPS, or IL-1α (Student's t test).

higher concentrations (10^{-8}–10^{-6} mol per liter) attenuated secretion. This has also been observed in rat vascular cells (Ishimitsu *et al*, 1994; Isumi *et al*, 1998). TPA, when administered over a prolonged period, inhibits protein kinase C activity (Nishizuki, 1998) and the dual affect of TPA is thought to occur via the protein kinase C pathway utilizing AP-1 and AP-2 regulatory sites in the 5′ region of the AM gene (Ishimitsu *et al*, 1994). The effect of TPA on AM secretion from oral and skin keratinocytes suggests that protein kinase C plays a part in the regulation of AM gene expression. In contrast to this, forskolin and ACTH, agents that stimulate cAMP production, and the cAMP analog, di-butyryl cAMP, all significantly inhibited AM secretion from skin and oral keratinocytes. The inhibitory effect with ACTH was also observed at the gene transcription level. The AM gene has cAMP response element in its 5′-upstream region and it is possible that the cAMP pathway partially regulates AM gene activation in keratinocytes of the skin and oral cavity.

Two cytokines were found to inhibit AM release from both oral and skin keratinocytes: TGF-β1 and IFN-γ. TGF-β1 is produced by a variety of cells in mucosa, including keratinocytes and it is thought to play an important part in downregulating the immune response and by inhibiting cell proliferation (Matsumoto *et al*, 1990). It is also important in the induction of IgA (rather than IgG or IgM synthesis) from B cells in mucosa (Coffman *et al*, 1989) and induces the expression of αeβ7 on intra-epithelial lymphocytes, which facilitates their interaction with epithelium (Cepek *et al*, 1993). Inhibition of AM production by keratinocytes is thus consistent with its immunosuppressive function. IFN-γ on the other hand is produced by activated T cells and high levels are produced in the oral mucosa and skin in inflammatory mucocutaneous disease (Palliard *et al*, 1988) where it is thought that it may be important in the induction of ICAM-1 on keratinocytes and endothelial cells. Such ICAM-1 expression is associated with migration of leukocytes from peripheral blood through vascular endothelium and also into epithelium (Griffiths and Nickoloff, 1989; Walton *et al*, 1998) and hence is important in facilitating the immune response. Why IFN-γ should inhibit AM production by keratinocytes is not clear but indicates that *in vivo* secretion will be modified by a complex interplay between differing factors.

Thrombin was a potent stimulator of AM synthesis from skin keratinocytes but its effects were much less marked on oral keratinocytes. Thrombin plays a part in the upregulation of the immune response by stimulating endothelial cells to express adhesion molecules such as ICAM-1 and release cytokines (Kaplanski *et al*, 1997). Our findings suggest that it may also upregulate the immune response particularly in the skin.

All steroid hormones tested stimulated a moderate increase in the secretion of AM from skin keratinocytes and similar effects have been reported on vascular cells (Sugo *et al*, 1994b, 1995a, >b; Imai *et al*, 1995; Minamino *et al*, 1995). Receptors for glucocorticoids are present in the cytoplasm and nuclei of skin keratinocytes (Serres *et al*, 1996) and upon binding they dimerize and translocate to the nucleus where they bind to glucocortocoid response elements on glucocorticoid response genes (Barnes, 1998). The 5′ regulatory region of the AM gene has a glucocortocoid response element and a study by Imai *et al* (1995) demonstrated specific effects of glucocorticoid on AM gene transcription in endothelial cells. Interestingly, steroid hormones had no statistical effect on oral keratinocytes.

At present the reason for these differences in the regulation of AM secretion from skin and oral keratinocytes are not clear. Receptors for IL-1, TNF, and so-called Toll receptors that bind LPS share a common intracellular pathway, which results in the translocation of the transcription factor NF-κB from the cytoplasm to the nucleus (see Murphy *et al*, 2000 for review). NF-κB is part of a highly conserved family and plays a key part in host defense. It is important in the induction of inflammatory cytokines, such as IL-1 and TNF-α, chemokines, including IL-8 and monocyte chemotactic protein-1, as well as the induction of adhesion molecules ICAM-1, vascular cell adhesion molecule-1, and E-selectin (Barnes and Karin, 1997). As IL-1, TNF, and LPS induce AM production in oral keratinocytes, our results suggest NF-κB may also be important in the regulation of its production; however, skin keratinocytes also respond to IL-1, TNF, and LPS by producing cytokines and chemokines (Kondo, 1999) and presumably by NF-κB, and it is not clear why they also do not produce AM. Presumably, there is a difference at the genetic level in the control of AM secretion between skin and oral keratinocytes but this is yet to be investigated.

In conclusion, this study has shown that there are interesting differences in the regulation of AM secretion between oral and skin keratinocytes and that oral keratinocytes are particularly responsive to the effects of proinflammatory cytokines and bacterial LPS. This raises the possibility that there are functional differences in the role of AM between the oral mucosa and skin.

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