Retinoids Downregulate Vascular Endothelial Growth Factor/ Vascular Permeability Factor Production by Normal Human Keratinocytes

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Normal human keratinocytes (KC) are a prominent source of vascular endothelial growth factor (VEGF)/ vascular permeability factor (VPF), both *in vivo* and in tissue culture. In this report we have investigated the influence of retinoids, which are used to treat several skin diseases, on VEGF/VPF production by KC. All-trans retinoic acid (RA), 13-cis RA, and all-trans retinol reduced VEGF/VPF secretion by KC in primary cultures by a mean ± SD of 58 ± 25%, 46 ± 21%, and 54 ± 20%, respectively, compared with control values. Reductions were observed at concentrations as low as 10^{-10} M for all-trans RA, a level that is easily reached *in vivo* during retinoid treatment. The reduction in VEGF/VPF protein by 10^{-6} M all-trans RA was paralleled by a strong downregulation of VEGF/VPF mRNA levels. In contrast to normal KC, all-trans RA had no effect on the HaCaT keratinocyte cell line, and it stimulated VEGF/VPF release by the epidermoid carcinoma cell line A431 2-fold. Our data demonstrate that retinoids are potent inhibitors of VEGF/VPF production by normal human KC. Downregulation of VEGF/VPF production in these cells by retinoids may contribute to the therapeutic effects or retinoids in diseases that are accompanied by angioproliferation, such as psoriasis. *Key words: angiogenesis/cytokines/endothelial cells/psoriasis. J Invest Dermatol 111:907–911, 1998*

Vascular endothelial growth factor (VEGF) is a basic, heparin-binding glycoprotein that occurs in four isoforms that include 121, 165, 189, and 201 amino acids, respectively (Houck *et al*, 1991; Tischer *et al*, 1991). Besides acting as a potent mitogen for endothelial cells *in vivo* and *in vitro*, and playing a central role in angiogenesis, VEGF increases microvascular permeability; hence, it is also referred to as vascular permeability factor (VPF) (for review see Dvorak *et al*, 1995; Ferrara and Davis-Smyth, 1997).

We and others have shown that normal human epidermal keratinocytes (KC) express the three major splice variants of VEGF/VPF *in vitro* (Detmar *et al*, 1994; Ballaun *et al*, 1995) and *in vivo* (Weninger *et al*, 1996). Upregulation of VEGF/VPF mRNA and protein *in vivo* is known to occur during wound healing (Brown *et al*, 1992), and in psoriasis (Detmar *et al*, 1994), contact dermatitis, bullous skin diseases (Brown *et al*, 1995a, b), common warts, and squamous cell carcinomas (Weninger *et al*, 1996). Rather than representing a secondary phenomenon, KC-derived VEGF/VPF probably contributes actively to the pathogenesis of these diseases *via* the induction of permeability in dermal vessels, through its chemotactic action on inflammatory cells and endothelial cells (Clauss *et al*, 1990; Koch *et al*, 1994; Gruber *et al*, 1995) and through its angiogenic effect.

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Abbreviations: KC, keratinocytes; RA, retinoic acid; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

Its central role in angiogenesis makes VEGF/VPF an important target for pro- and anti-angiogenic therapy. Concerning the regulation of VEGF/VPF expression, various stimuli, including hypoxia and exposure to certain cytokines such as epidermal growth factor, transforming growth factor-α (TGFα), and interleukin-6, have been found to upregulate VEGF/VPF production (Dvorak *et al*, 1996; Ferrara and Davis-Smyth, 1997). Suppression of VEGF/VPF expression has so far only been observed for corticosteroids (Harada *et al*, 1994; Palacio *et al*, 1997).

Systemic administration of retinoids is used widely to treat psoriasis, ichthyoses, acne, and rosacea, and topical administration has been used to treat acne and photoaging. In addition to a variety of other biologic activities, including effects on differentiation and proliferation of KC (for extensive reviews see Chambon, 1996; Fisher and Voorhees, 1996), retinoids have been shown to inhibit angiogenesis in several experimental systems. They are able to abrogate tumor-associated angiogenesis *in vivo* (Majewski *et al*, 1994), switch the phenotype of tumor cells *in vitro* from angiogenic to anti-angiogenic (Lingen *et al*, 1996a), and inhibit vessel ingrowth into the chorioallantoic membrane of the chick (Oikawa *et al*, 1989, 1993). Such anti-angiogenic effects of retinoids may result from altered endothelial cells activities, such as the inhibition of endothelial cell migration (Lingen *et al*, 1996b), as well as from modulated production of factors that stimulate or inhibit vessel growth (Lingen *et al*, 1996b).

VEGF/VPF overproduction by KC has been implied in the characteristic vascular changes commonly observed in psoriasis (Detmar *et al*, 1994). Because retinoids are a mainstay of anti-psoriatic therapy, we examined their effects on VEGF/VPF production by KC. In this report we demonstrate that all-trans retinoic acid (RA), 13-cis RA, and all-trans retinol suppress secretion of VEGF/VPF by KC in primary cultures, but not by KC-derived cell lines.

MATERIALS AND METHODS

Cell culture Human neonatal foreskin KC derived from five different donors were purchased from PromoCell (Heidelberg, Germany) or Clonetics (San Diego, CA). KC were cultured under low-Ca^{2+} conditions (0.15 mM) in serum-free KC growth medium consisting of KC basal medium (KBM) supplemented with human recombinant epidermal growth factor (0.1 ng per ml), bovine pituitary extract, insulin (5 μg per ml), hydrocortisone (0.5 μg per ml), and gentamycin/amphotericin B (50 μg per ml, 50 ng per ml) (all from either PromoCell or Clonetics) at 37°C in 5% CO_2. Cells were routinely passaged at 60%–80% confluence using each manufacturer's detachment solution (0.05% trypsin/0.01% ethylenediamine tetraacetic acid). All experiments were carried out between passages 2 and 5.

The epidermoid carcinoma cell line A431, the immortalized keratinocyte cell line HaCaT (passage number > 35) were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (all GIBCO BRL, Gaithersburg, MD).

Retinoids All-trans RA, 13-cis RA, and all-trans retinol were purchased from Sigma (St. Louis, MO). Stock solutions were prepared in dimethylsulfoxide (DMSO, 10^{-2} M) and stored protected from light at –20°C. At the time of use, working solutions were prepared with KBM or with RPMI-1640 supplemented with 2 mM L-glutamine.

Stimulation experiments For all experiments, 3×10^5 primary KC were seeded into 6 well plates (Costar, Cambridge, MA) in 2 ml of KC growth medium and allowed to attach overnight. After 12–16 h, KC growth medium was changed to either KBM or KBM supplemented with TGFα (100 ng per ml, PBH, Hannover, Germany), with various concentrations of the respective retinoids, with TGFα plus all-trans RA, or with DMSO alone.

For experiments with the cell lines, 3×10^5 cells were allowed to attach overnight in RPMI-1640/10% fetal bovine serum. Then the medium was changed to RPMI-1640, without fetal bovine serum, containing the indicated reagents.

Supernatants were collected after 24 h and centrifuged sequentially at 700 and 17,600 × *g* to remove cellular debris. The supernatants were stored frozen at –20°C until further analysis.

Assessment of cytotoxicity and cell proliferation After collecting the supernatants, cells were detached and viability tested with Trypan-blue. Cell proliferation was assessed with the colorimetric assay EZ4U as described by the manufacturer (Biomedica, Vienna, Austria). This test measures the reduction of tetrazolium salts into intensely colored formazan derivatives, which occurs only in living cells (Scudiero *et al*, 1988). Briefly, 1×10^4 cells were seeded into 96 well plates with KBM or KBM plus stimulants. Four hours prior to measurement, the tetrazolium-salt was added. Extinction of the cell culture supernatant was measured on an enzyme linked immunosorbent assay (ELISA) reader.

VEGF/VPF immunoassay Cell culture supernatants were analyzed using a commercial VEGF-ELISA kit (CYTElisa-VEGF, CYTimmune Sciences, College Park, MD) according to the manufacturer's instructions.

Northern blot analysis KC were cultured at 60% confluence in 25 cm^2 culture flasks (Costar) in either KBM or KBM supplemented with TGFα (100 ng per ml), 0.01% DMSO, or all-trans RA at several concentrations. After 4 or 8 h cells were lyzed and total RNA extracted using the RNAzol B method (CINNA/Biotecx Lab, Houston, TX). RNA was size fractionated in 1% agarose gels containing 1.48% formaldehyde and transferred to nylon membranes (Nytran, Schleicher and Schüll, Dassel, Germany). Northern hybridization with either a VEGF/VPF or a GAPDH cDNA probe was performed under stringent conditions as described previously (Weninger *et al*, 1996).

Statistical analyses Treatment effects on KC in primary cultures were compared by analysis of variance. The Student's t test was used to calculate differences in the cell line experiments. Significance was established at the level of $p < 0.05$.

RESULTS

Effects of retinoids on the proliferation and viability of KC in primary culture and of KC-derived cell lines The addition of all-trans RA, 13-cis RA, or all-trans retinol to KC cultures at concentrations > 10^{-5} M lead to significant cell death within 24 h. Below 10^{-5} M, cell viability remained above 95% and cell morphology, as judged by light microscopy, was not altered, i.e., the primary keratinocytes appeared small and cobblestone-shaped (Boyce and Ham, 1983). All subsequent experiments were performed at retinoid concentrations of 10^{-6} M or less. Using a colorimetric proliferation assay, we found that KC proliferative activity stayed within a range of ±10% when cells cultured in KBM were compared with those cultured in KBM supplemented with the respective retinoids or DMSO, confirming previous studies (data not shown, Varani *et al*, 1989). In some experiments, these data were also confirmed by manual cell counts and measurement of total cellular protein. Also, no effect was observed on the proliferation of A431 cells and HaCaT cells (data not shown).

Retinoids suppress VEGF/VPF secretion by KC in primary cultures TGFα, a well-known inducer of VEGF/VPF secretion in KC, was used as a positive control in each experiment. The mean ± SD stimulatory effect of TGFα as compared with medium alone was 333% ± 152% ($p < 0.001$, data not shown). Incubation of KC with all-trans RA resulted in a dose-dependent reduction of VEGF/VPF in culture supernatants as assessed by ELISA. A typical dose-response curve is shown in **Fig 1(*a*)**. Strong reduction of VEGF/VPF secretion by KC was observed with concentrations of all-trans RA equal to and below 10^{-6} M. A total of 13 individual experiments with 10^{-6} M all-trans RA was carried out, of which seven were run in triplicate. All-trans RA suppressed VEGF/VPF production to a mean ± SD of 58 ± 25% compared with the DMSO control ($p < 0.001$). Similar data were obtained when 13-cis RA (five individual experiments) or all-trans retinol (four individual experiments) were examined (**Fig 1*b***), i.e., VEGF/VPF levels were reduced to 46 ± 21% ($p < 0.001$) and 54 ± 20% ($p < 0.001$) of control values, respectively.

To determine whether retinoids also influenced TGFα-induced upregulation of VEGF/VPF secretion, KC were incubated simultaneously with TGFα (100 ng per ml) and all-trans RA (10^{-6} M). A significant decrease (mean ± SD, 75% ± 11% of control, $p < 0.001$) was found after 24 h of incubation (**Fig 2**).

All-trans RA upregulates VEGF/VPF secretion by A431 cells and does not affect its secretion by HaCaT cells In contrast to what we observed for KC in primary culture, all-trans RA induced VEGF/VPF production strongly and in a dose-dependent manner in the epidermoid carcinoma cell line A431 (**Fig 3*a***). The mean ± SD upregulation of VEGF/VPF observed in five independent experiments was 2.2 ± 0.15-fold ($p < 0.01$, **Fig 3*b***). No significant effects on VEGF/VPF protein secretion were observed for the keratinocyte cell line HaCaT (**Fig 3*c***).

All-trans RA downregulates VEGF/VPF mRNA expression in KC When we examined VEGF/VPF mRNA expression in KC exposed to 10^{-6} M all-trans RA, we observed a decrease to about 25% of levels obtained in control cultures incubated with 0.01% DMSO alone after 4 h (**Fig 4**). This effect was still present after 8 h. In some experiments upregulation of VEGF/VPF mRNA was observed in KC cultured in 0.01% DMSO, compared with those cultured in KBM medium alone. This effect was not paralleled by increased VEGF/VPF secretion by the cells.

The effect of all-trans RA was also studied in A431 cells. Clear upregulation of VEGF/VPF mRNA was observed at concentrations between 10^{-6} and 10^{-8} M after 4 h (data not shown).

DISCUSSION

In this study we demonstrate that retinoids downregulate spontaneous and TGFα-induced VEGF/VPF mRNA and protein production in primary cultures of human KC. VEGF/VPF secretion was reduced to 58%, 41%, and 52% by all-trans RA, 13-cis RA, and all-trans retinol, respectively. The inhibition of VEGF/VPF release was strongest at retinoid concentrations of 10^{-6} M, which can be reached during systemic therapy (Smith *et al*, 1992). The baseline release of VEGF/VPF differed among several batches of KC and among individual experiments using the same batch, despite the fact that culture conditions were otherwise identical. Differences in storing and shipping of the samples and resulting different plating efficiency might account for those deviations; however, because in each experiment retinoid-treated KC exhibited lower levels of VEGF/VPF secretion than did DMSO-treated control cells, regardless of the absolute amount of

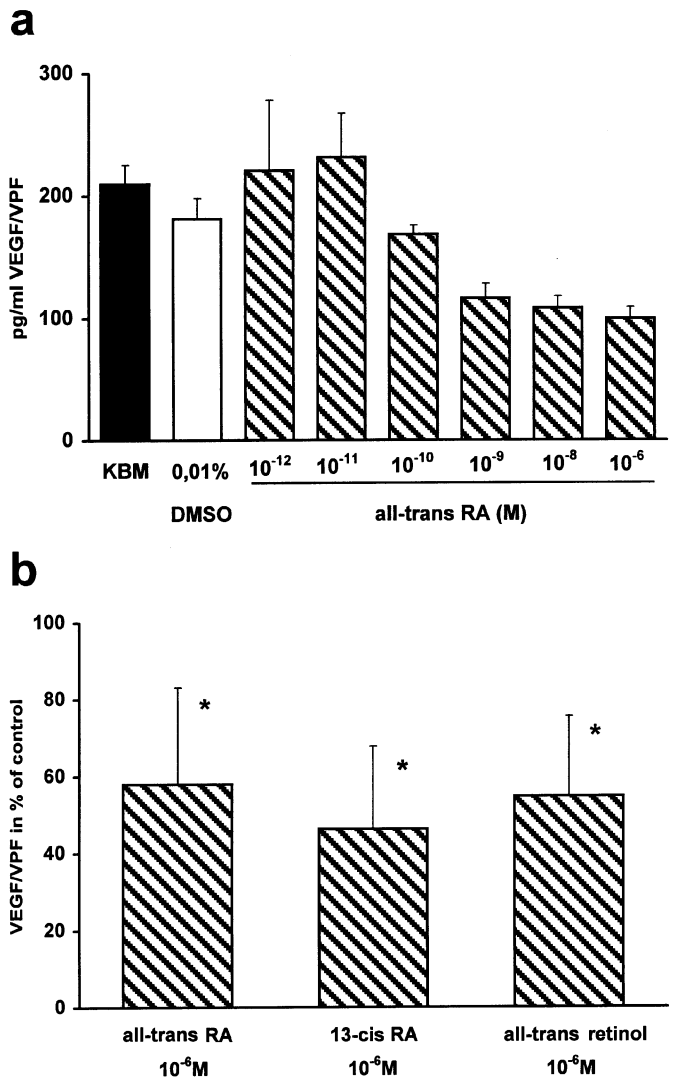

Figure 1. VEGF/VPF secretion by KC is suppressed by all-trans RA, 13-cis RA, and all-trans retinol. KC were incubated for 24 h with the indicated concentrations of retinoids as described in *Materials and Methods*. Supernatants were analyzed using a VEGF/VPF-specific ELISA. For (*a*), KC were cultured in either KBM alone (black bar), KBM supplemented with 0.01% DMSO without (open bar), or different concentrations of all-trans RA (hatched bar). Each bar represents the mean ± SD VEGF/VPF concentration in pg per ml of triplicate assays. In (*b*), KC were incubated with either 10^{-6} M all-trans RA, 10^{-6} M 13-cis RA, or 10^{-6} M all-trans retinol. Each bar represents the mean ± SD inhibition in percentage of the DMSO control. For all-trans RA, 13-cis RA, and all-trans retinol 13, 5, and 4, respectively, independent experiments were carried out, and at least three of each group were run in triplicate. *$p < 0.001$ (ANOVA).

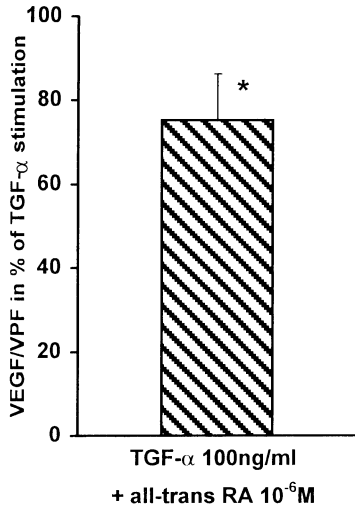

Figure 2. All-trans RA reduces TGFα-induced VEGF/VPF upregulation by primary human KC. KC were incubated simultaneously with TGFα (100 ng per ml) and all-trans RA (10^{-6}M) for 24 h. Supernatants were analyzed using a VEGF/VPF-specific ELISA. The bar represents the mean ± SD inhibition in percentage of control. Five individual experiments in triplicate were carried out. *$p < 0.001$ (ANOVA).

VEGF/VPF, differing baseline values do not interfere with the conclusions on the inhibitory effects of retinoids.

The downregulatory effect of all-trans RA on VEGF/VPF production was also observed when KC were cultured in the presence of TGFα, a strong inductor of this cytokine (Detmar *et al*, 1994). Given that TGFα is strongly upregulated in psoriatic epidermis (Elder *et al*, 1989), the ability of retinoids to reduce the effects of TGFα on VEGF/VPF production *in vitro* argues for its potential to interrupt the cycle of paracrine VEGF/VPF induction in psoriasis.

In addition to psoriasis, systemic retinoids are widely used as an effective treatment for rosacea (Plewig, 1993). The cardinal signs of this disorder, i.e., facial edema, erythema, and telangiectasias (Plewig, 1993; Wilkin, 1994), are also highly suggestive of a pathogenic role for VEGF/VPF. Therefore, it is tempting to speculate that the reduction of these signs after isotretinoin treatment might be due, at least in part, to the inhibited VEGF/VPF production. We are currently investigating the production of VEGF/VPF by KC in this disease.

Topical retinoid therapy is used to reverse some of the effects of photoaging (Gilchrest, 1992; Kang *et al*, 1997). After long-term application of tretinoin, skin architecture is dramatically changed, and increased epidermal thickness, deposition of extracellular matrix proteins, and increased vascularity of the dermis can be observed (Fisher *et al*, 1991; Gilchrest, 1992; Griffith *et al*, 1995; Kang *et al*, 1997). These findings are at first glance contradictory to our results on the inhibition of VEGF/VPF production by retinoids; however, the effects of long-term retinoid application on epidermis certainly differ from those of short-term exposure of KC in tissue culture. In addition, topical retinoids have different effects on the epidermis than do systemic retinoids. Whereas the former induce KC proliferation, resulting in a thicker epidermis, the latter lead to epidermal thinning in diseases such as psoriasis as a consequence of a decreased proliferation of KC (Gilchrest, 1992; Griffith *et al*, 1995; Gottlieb *et al*, 1996). Thus, even if VEGF/VPF production on a per cell base should be reduced after topical retinoid treatment of photoaged skin, an increase in keratinocyte numbers might lead to a relative increase in secreted growth factors, including VEGF/VPF. Future studies will be necessary to verify this scenario, however.

In contrast to what we observed for primary KC, no alteration of VEGF/VPF production by retinoids was found with the KC-derived cell line HaCaT, and VEGF/VPF mRNA and protein were even upregulated 2-fold in A431 cells. Concerning the possible relevance to our understanding of the therapeutic effects of retinoids, the data obtained with the cell lines suggest that the anti-angiogenic effects of retinoids *in vivo* might not be generally due to downregulated VEGF/VPF production. As has been suggested previously, these effects may be attributable to a direct action on endothelial cells and to the induction of anti-angiogenic molecules instead (Lingen *et al*, 1996a, b).

Retinoids act by binding to retinoic acid receptors and retinoid X receptors (for review see Chambon, 1996). These heterodimeric receptor complexes bind to the retinoic acid responsive element within promoter regions and stimulate the transcription of retinoid responsive genes (Chambon, 1996). Besides transcriptional activation, retinoids can also repress transcription, a process that is referred to as transrepression (Chambon, 1996; Fisher and Voorhees, 1996). In contrast to transcriptional activation, transrepression does not involve interaction of the retinoic acid receptors/retinoid X receptors complex with the retinoic acid responsive element. Rather, it depends on the transcription factor AP-1, which is composed of c-jun and c-fos proteins (Chambon,

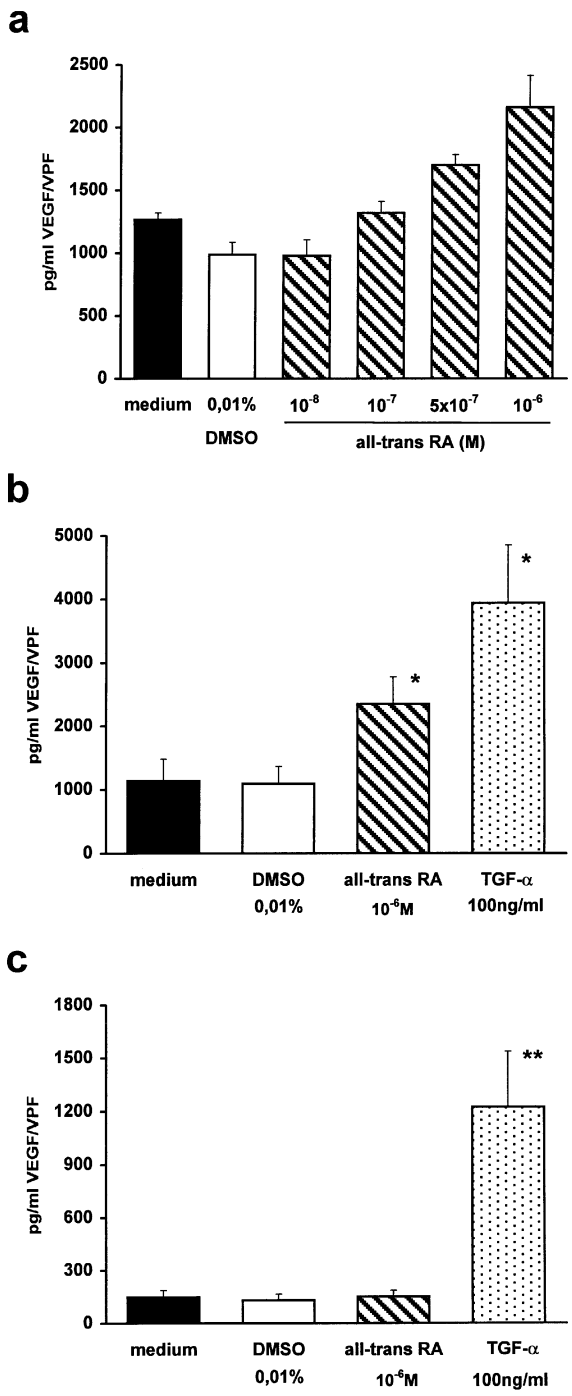

Figure 3. All-trans RA upregulates VEGF/VPF secretion by A431 cells, but not by HaCaT cells. A431 cells (*a*, *b*) or HaCaT cells (*c*) were incubated in RPMI-1640 alone (black bars), or supplemented with either 0.01% DMSO (open bars), all-trans RA (hatched bars), or TGFα (100 ng per ml, punctata bars) for 24 h. Supernatants were analyzed using a VEGF/VPF-specific ELISA. Bars represent mean ± SD VEGF/VPF concentration in pg per ml. Part (*a*) shows a dose-response curve with all-trans RA performed in triplicate. In (*b*) and (*c*) each bar is the mean ± SD level of VEGF/VPF secretion of five and three, respectively, individual experiments performed in triplicate. The values were compared with the Student's t test. *$p < 0.01$, **$p = 0.03$.

1996; Fisher and Voorhees, 1996; Karin *et al*, 1997), and it is thought to be mediated either by physical interaction of retinoid receptors with c-jun and c-fos, or, alternatively, by their competition for a possible third binding partner (Chambon, 1996; Fisher and Voorhees, 1996). Because the VEGF/VPF promoter contains at least one AP-1 site (Tischer *et al*, 1991), transrepression may play a role in the downregulation of VEGF/VPF by retinoids in KC in primary culture. This

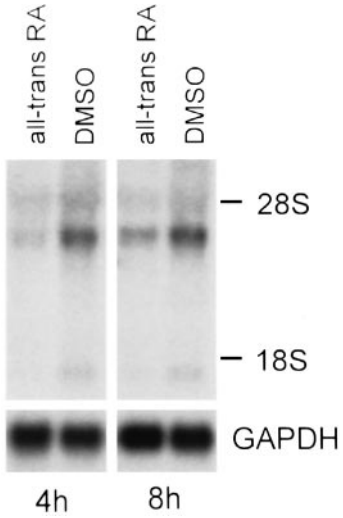

Figure 4. All-trans RA suppresses VEGF/VPF mRNA expression in KC. KC were incubated in KBM supplemented with 0.01% DMSO with or without 10^{-6} M all-trans RA. Total RNA was isolated after 4 h and 8 h, subjected to agarose gel electrophoresis, transferred to a nylon membrane, and analyzed by northern hybridization for VEGF/VPF (upper panel) or GAPDH (lower panel) mRNA expression.

assumption is supported by the observed decrease in VEGF/VPF mRNA after retinoid treatment of KC; however, we cannot formally exclude the possibility that reduced mRNA stability is also involved.

Because transrepression is influenced by the amount of retinoid receptor proteins present and by the level of AP-1 activation (Chambon, 1996; Fisher and Voorhees, 1996), different levels of these molecules in KC, HaCaT, and A431 cells may explain the different effects of retinoids observed.

In summary, we have demonstrated that retinoids downregulate VEGF/VPF mRNA expression and protein production in primary culture of KC. Based on these findings we suggest that the therapeutic action of retinoids, as observed in inflammatory skin disorders associated with hypervascularization such as psoriasis, is due, at least in part, to the suppression of VEGF/VPF secretion by KC.

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