The proliferative effect of all-trans retinoic acid during wound healing: A compromised effect in the presence of dimethyl sulphoxide

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Introduction

During wound healing, interactions between the cells resident in skin and the infiltrating cells trigger the release of cytokines and growth factors such as transforming growth factor-α (TGF-α), epidermal growth factor, keratinocyte growth factor and vascular endothelial growth factor[1-4]. However, in addition to these factors, external factors have been reported to affect the healing process as well. One such external factor important in wound healing is retinoic acid[5-10].

Several reports have shown that *all-trans* retinoic acid (ATRA), the active metabolic component of retinoic acid[7], is beneficial to wound healing[11-14]. ATRA has been shown to increase wound closure and facilitate repair of the epithelium in Wistar-Albino rats as well as increasing collagen synthesis, angiogenesis and formation of new granulation tissue[13]. In addition, ATRA is said to reverse the non-healing effects of corticosteroids[12,14], promotes the healing of impaired wounds[15] such as in diabetes mellitus[16].

However, disparate reports in the literature contradict the described beneficial effects of ATRA on wound healing. For example, Kitano *et al.*[17] reported a non-significant effect of ATRA on wound closure in normal mice, but a significant increase in closure in diabetic mice. Furthermore, the continuous treatment of wounds with ATRA, inhibited re-epithelisation[18], disrupted collagen production and decreased the breaking strength of skin[8]. It was thus suggested by Muehlberger *et al.*[8] that ATRA did not have an effect on unimpaired wounds, but was beneficial only in patients with impaired vitamin A metabolism, or patients with chronic non-healing wounds.

Similarly, in *in vitro* studies, ATRA has been reported to decrease cell proliferation and procollagen production in human dermal fibroblasts[5] and to inhibit cell proliferation in 3T3 fibroblast and two transformed fibroblasts cell lines (3T12-3 and 3T3-B-SV40)[19]. In addition, in the latter experiment, ATRA caused increased contraction of the collagen gel in which the fibroblasts were seeded. It was thus concluded that ATRA promoted contraction of collagen but it was uncertain whether ATRA promoted cell proliferation[19].

Reports have also shown that the *in vitro* effect of ATRA may differ from its *in vivo* effects. Fisher and Voorhees[7] compared the effect of ATRA on keratinocytes and concluded that ATRA promoted proliferation of keratinocytes in the basal layer of the epidermis *in vivo* but not *in vitro.* The basal cells, in response to ATRA treatment, migrated to the suprabasal layer before terminally differentiating and losing their ability to proliferate. *In vitro,* however, differentiation of the keratinocytes resulted in the arrest of cell proliferation[7] or the cells undergoing functional differentiation characterised by the expression of markers for differentiation[6,7].

According to reports in the literature, ATRA significantly inhibits differentiation in cells at a concentration of 1μM of

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ATRA[6,7]. It was for this reason that Eckhart *et al.*[9] regarded 1µM ATRA as the "physiologically relevant concentration" for studies on wound healing in skin. This is thus the concentration of ATRA frequently utilised in several studies on skin cell types[10,20-22].

While contradictions on the effect of ATRA on wound healing still exist in the literature, retinoic acid is approved by the Food and Drug Administration (FDA) and is still widely used for the treatment of acne and photo-ageing[23,24]. It is also used as pretreatment against skin injuries before laser therapy[15,25]. The contradictions in studies examining the effect of ATRA are said to be due to the biomolecular mode of action of retinoic acid[26,27]. One of the diverse, yet unknown, effects may possibly be indirect stimulation or release of biological mediators which could be responsible for promoting or inhibiting any of the phases of wound healing[26-28]. A possible interaction of ATRA with other co-activators released by cells or whose release is regulated by ATRA could augment or diminish the effects of ATRA[10,29]. It is also possible that ATRA may elicit numerous biological functions that could be difficult to link to a specific gene transcription[30,31].

In addition, it is also possible that dimethyl sulphoxide (DMSO) which is often a diluent of ATRA could 'mask' the effect of ATRA as DMSO, itself, has been reported to stimulate wound healing[32]. Topical application of DMSO on a vinorelbine extravasation-induced injury on rat skin significantly promoted the reduction of the size of the lesion and the healing time when compared to the control[33]. However, there are similarly contradictory reports on the effect of DMSO on wound healing. Goldblum *et al.*[34], while investigating the dermal/epidermal healing of partial thickness wounds on Yorkshire pigs treated topically with DMSO, reported that it had no beneficial effect on wound healing. However, there was a significant reduction in the repopulation of cells in the wound region of adult male rats treated with DMSO compared to the control during the inflammatory stage, but no significant difference was observed between the treatment groups during the phase of healing[35].

The relationship between the effects of ATRA and DMSO may thus be more complicated than what is known. In order to elucidate this in a controlled *in vitro* situation, we investigated the effect of ATRA on proliferation of cells during wound healing in an *in vitro* mimic of skin composed of two cell lines. In order to ascertain if DMSO was compromising the effect of ATRA on a particular cell line in the co-culture, its effect and that of ATRA was similarly investigated on the cell lines cultured separately *in vitro*.

Materials and methods

Cultures of cell lines

An HaCaT cell line[36] (donated by the German Cancer Research Centre) and an HDF cell line (ATCC, USA) were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Lonza, South Africa) and Iscove's Modified Dulbecco's Medium (IMDM) (Lonza, South Africa) respectively supplemented with 10% foetal bovine serum (FBS) (Lonza, South Africa) and a 0.1% mixture of streptomycin and penicillin (Sigma, South Africa) in phosphate buffer saline (PBS). All incubation of the cultures in this study was at 37° C in a 5% CO_2 in an air-humidified incubator.

Culturing of co-cultures

The HDF cell line was seeded at a concentration of 1 X 10^4 cells per ml of IMDM supplemented with 10% FBS into a six-well culture plate and incubated for five days. After five days, the medium was aspirated and then the HaCaT cells, at a concentration of 1 X 10^6 cells per ml of DMEM supplemented with 10% FBS, was carefully seeded on to the HDF cultures and then incubated as above.

Concentration of all-trans retinoic acid (ATRA)

ATRA (Sigma, South Africa) at a concentration of 1µM was used in all the experiments due to studies describing this as a physiologically relevant concentration at which terminal differentiation of keratinocytes is inhibited[6,7,9,37]. ATRA at this particular concentration was reconstituted in DMSO (Sigma, South Africa). DMSO (vehicle control) was used at a concentration of 0.0015% while the untreated control had medium alone. All media were also supplemented with 10% FBS.

Colourimetric cell proliferation assay

The proliferative effect of ATRA was initially investigated individually on the two cell lines to obtain baseline information. The HaCaT or HDF cells were seeded at a concentration of 1 X 10^4 cells per ml per well of a 96-well microplate in triplicate either in DMEM medium or IMDM medium according to the cell line. At 72 hours after seeding, the cultures were treated with ATRA (n=6) or placed in the respective control medium, vehicle (n=6) or untreated control (n=6), for 24 hours. After the treatment of the cells for 24 hours, the cell proliferative activity was determined using a CellTiter 96 Aqueous® reagent (Promega, South Africa), a colourimetric cell proliferation kit. The absorbance, which gives an indication of cell proliferation expressed as a percentage of the untreated control, was measured at 490 nm with an Anthos 2010 spectrophotometer.

Scratch assay for cell proliferation analysis

The HaCaT cultures or the co-cultures were seeded on to a cover slip placed inside each well of a six-well culture plate. The cultures were scratched or "wounded" as described by Buth *et al.*[38] and treated for 24 hours. After 24 hours of treatment, both the HaCaT cultures and the co-cultures were immunolabelled with Ki-67 antibody (a cell proliferation marker). The number of the immunolabelled nuclei in both the HaCaT cultures and the co-cultures for 0 hour and 24 hour [for the ATRA treatment (n=4), untreated control (n=4) and vehicle control (n=4)] treatment durations was determined by counting these actively proliferating nuclei located around the wound region[39]. For ease of counting and validation of the counted cells, five photographs were taken in series at every 3-mm interval around (achieved by moving the calibrated stage of the microscope) the margin of the wound region with X10 objective of a Zeiss inverted microscope attached to a digital camera. Thus the area of field was standard in all the photographed regions.

A total of 10 photographs (five photographs from each side of the wound) were taken per culture. The Ki-67 positively immunolabelled nuclei on each of the photographs were manually counted with the use of a grid and a cell counter using the ImageJ software from the NIH in the United States. The Ki-67 immunolabelled nuclei were counted again after a two-week interval under blinded conditions. The estimated number of the Ki-67 positively immunolabelled nuclei at 0 hour and 24 hours (for the ATRA treatment, the untreated control and the vehicle control) for both the HaCaT cultures and the co-culture were compared.

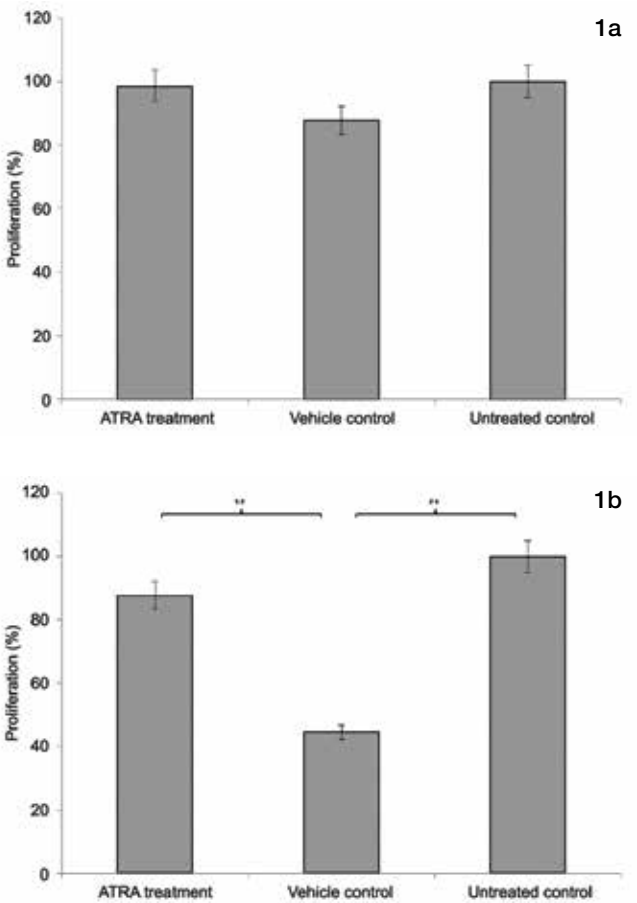

*Figure 1. Colourimetric cell proliferation assay of the HaCaT and the HDF cell lines. After the treatment of the cells for 24 hours, the HaCaT (a) or the HDF (b) cell line was incubated with 20µl CellTiter 96 Aqueous® reagent. The absorbance, which gives an indication of cell proliferation expressed as a percentage of the untreated control, was measured at 490 nm with a spectrophotometer. (Mean ± standard error, n=6, **p<0.01).*

Statistical analysis

Data was tested using a Mann-Whitney and a Kruskal-Wallis one-way analysis of variance test at a significant level of 5%.

Results

Proliferative effect of ATRA on the cell lines

Cell proliferation analysis using the colourimetric assay on the HaCaT cell cultures showed no significant difference in cell proliferation of the cultures treated with ATRA compared to the untreated control cultures (p=0.2980) or the vehicle control cultures (p=0.1735) (Figure 1a). Similarly, there was no significant difference in cell proliferation when the vehicle control cultures were compared with the untreated control cultures (p=0.8102).

In the HDF cells (Figure 1b), cell proliferation was decreased in the cultures treated with ATRA, but this decrease was not significantly different when compared with the untreated controls (p=0.1495). In addition, there was a significant inhibition of proliferation in the vehicle control when compared to the untreated control (p=0.0051) and the ATRA (p=0.0051).

Proliferative effect of ATRA during 'wound' closure

Positive proliferating cells in the HaCaT cultures and in the co-cultures of HaCaT and HDF cells, have dark labelled nuclei when immunolabelled with Ki-67 antibody (Figure 2). There was an increase in the number of actively proliferating cells around the 'wound' region in both the HaCaT cultures and the co-cultures from 0 hour to 24 hours in the cultures treated with ATRA and in those cultured in the relevant control media (Figures 3a and b).

At 24 hours (Figure 3a), there was no statistically significant difference in the number of actively proliferating cells around the 'wound' region in the HaCaT cultures when the cultures treated with ATRA and the two control cultures were compared. In the co-cultures at 24 hours (Figure 3b), there was a non-significant decrease in the number of actively proliferating cells around the 'wound' region in the co-cultures treated with ATRA when compared to the untreated control (p=0.6033). A significant decrease in the number of actively proliferating cells occurred in the vehicle control co-cultures when compared to the ATRA-treated co-cultures or the untreated control co-cultures (p=0.0020 or p=2.11E-05) respectively.

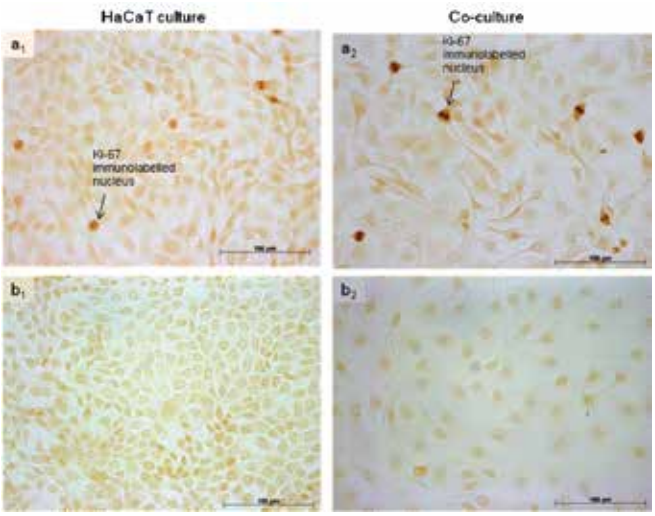

Figure 2. Photographic representations of HaCaT cultures and co-cultures of HaCaT and HDF cells immunolabelled with Ki-67 primary antibody. Photographs a_1 – a_2 represent the cultures with positive Ki-67 immunolabelled nuclei while b_1 – b_2 represent the cultures in which the Ki-67 primary antibody was omitted (negative control). Phase contrast microscopy, X40.

Discussion

The results of the present study showed that treatment of the HaCaT cultures with 1µM ATRA or with the vehicle control did not have an effect on cell proliferation. The non-proliferative effect of ATRA on the HaCaT cultures is in accordance with the report by Weninger *et al.*[21] on primary human keratinocyte cells. Weninger[21] also reported that 0.01% DMSO (used as the vehicle control) did not have an effect on cell proliferation in these cultures or on HaCaT cell lines and epidermoid carcinoma cell lines.

ATRA produced a noticeable, but insignificant inhibition of cell proliferation in the HDF cells when compared to the untreated control. There was similarly a significant decrease in cell proliferation in the HDF cultures grown in the vehicle control medium. The slight inhibition on proliferation by ATRA on the HDF cells in the present study compares with reports[5,40] which demonstrated inhibition of proliferation following treatment with 1µM ATRA of neonatal human dermal fibroblasts[5], and fibroblasts cells derived from normal human skin as well as from lesional and non-lesional skin of patients with psoriasis[40]. Similarly, it was reported that ATRA at concentrations of 0.1µM and 1µM significantly inhibited the proliferation of 3T3 fibroblasts as well as cell proliferation of two transformed fibroblast cell lines (3T12-3 and 3T3-B-SV40) embedded in a three-dimensional collagen gel. However, it is apparent that in the present study, the vehicle, DMSO, may be a confounding influence.

The number of actively proliferating cells in the HaCaT cultures and in the co-cultures did not differ when the

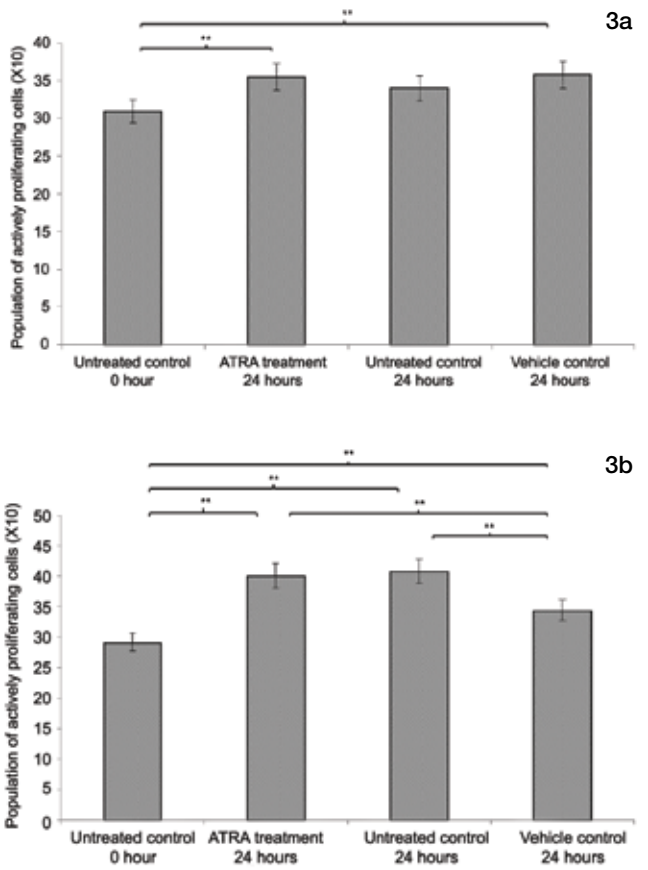

*Figure 3. Number of actively proliferated cells on the wound edge during closure of wound. After the treatment of the cultures for 24 hours post-wounding, the cultures were fixed for 24 hours and then incubated overnight at 4° C with a mouse monoclonal anti-proliferating cell protein Ki-67 primary antibody in 3% bovine serum albumin in TBS. The numbers of darkly labelled nuclei around the wound edge for the HaCaT cultures (a) and the co-cultures (b) were manually counted for each of the treatment groups with the use of a grid and a cell counter using the ImageJ software from the NIH in the United States. (Mean ± standard error, n=4, **p<0.01).*

ATRA treated and the untreated controls were compared. Interestingly, it has been reported that 1µM ATRA significantly induced, and at the same time, suppressed genes which are associated with the regulation of cell proliferation[10]. This was verified by determining the number of cells in ATRA-treated cultures at 24 and 48 hours following treatment[10]. The results, however, showed moderate cell proliferation at both 24 and 48 hours, but proliferation was significantly increased in the ATRA-treated cultures compared to the untreated cultures[10]. Despite observing a simultaneous expression and suppression of genes associated with cell proliferation, Lee *et al.*[10] concluded that ATRA promotes cell proliferation in keratinocytes. However, no proliferative effect of ATRA on the HaCaT, the HDF cell lines or the co-cultures was found in the present study.

The number of actively proliferating cells in the co-cultures grown in the vehicle control medium (DMSO) was statistically significantly decreased when compared to either the co-cultures treated with ATRA or grown in untreated control medium. However, no effect of the vehicle control medium was found in the HaCaT cultures. Thus, while DMSO appears to have had an inhibitory or "masking" effect on the co-cultures and the HDF cell line, it did not appear to have any effect on the HaCaT cell line alone. The reason for the difference in the proliferative effect of DMSO in the HDF cells and the HaCaT cells and thus on the co-cultures cannot be ascertained in the present study. Unfortunately, suitable reports in the literature for comparison of the effect of DMSO on cell proliferation in HaCaT and HDF cell lines, as well as in the co-cultures were not found. DMSO has been implicated in compromising the positive effect of ATRA on the proliferation of chick embryonic insulin cells *in vitro*[41]. In addition, DMSO was reported to be cytotoxic to 3T3 fibroblasts at concentrations above 1% but produced "negligible effects" at concentrations lower than 0.5%[42]. The extent of the "negligible effects" was, however, not elucidated in the latter report. This raises a question as to why DMSO is often used as the vehicle of choice for ATRA, despite the fact that its mode of action is still not fully understood. The present study thus suggests that DMSO had no effect on cell proliferation during 'wound' closure of the HaCaT culture, but that it appears to reduce/mask cell proliferation during 'wound' closure in the co-cultures as it may be exerting an effect on the HDF cell line, thus obfuscating the true proliferative effect of ATRA in wound closure.

Conclusions

The present study found that ATRA had no effect on cell proliferation in either of the individual cell lines used in this study or in the co-cultures. The DMSO (vehicle control) inhibited proliferation of the HDF cells as well as the co-cultures but did not appear to have an effect on the HaCaT cultures. It thus seems that the inhibitory effect of DMSO on proliferation appears to be directed towards the HDF cells as well as the co-cultures which contained HDF cells. In light of the failure of ATRA to promote proliferation during wound closure of the HaCaT culture and the co-culture in the present study, it would seem that the activity of ATRA was compromised in the presence of DMSO. This may also substantiate why there is controversy in the literature

regarding the effect of ATRA, as DMSO is often the diluent of choice for ATRA.

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