Short- and long-term effects of acetylsalicylic acid treatment on the proliferation and lipid peroxidation of skin cultured melanocytes of active vitiligo

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ABSTRACT

Objective: Recent in vitro and in vivo studies have shown that the nonsteroidal anti-inflammatory agent, acetylsalicylic acid (ASA) or aspirin has antioxidant properties on various cell lines and tissues. Hence, the aim of the present study is to investigate the effects of ASA at 2 different concentrations (75 and 300µg/ml) on the proliferative capacities and lipid peroxidation of in vitro skin cultured melanocytes obtained from patients with active vitiligo.

Methods: The present work was carried out from February 2001 through to November 2001, at the Vitiligo Unit, King Abdul-Aziz University Medical Center, Jeddah, Kingdom of Saudi Arabia. Employing methods described in this section, cryopreserved primary cultured melanocytes that were originally cultured from skin biopsies of normal healthy individuals and patients with active vitiligo (n=7), were subcultured to confluence. The malondialdehyde (MDA) concentrations in the cell culture medium were determined at 6 hours and 21 days following cultured melanocytes treatment with ASA (75 and 300µg/ml). Also, the number of viable melanocytes was determined 21 days following the treatment of melanocytes with ASA (75 and 300µg/ml).

Results: Following ASA treatment at 75µg/ml, the cultured melanocytes from the normal and active vitiligo donors showed significant increase in the proliferative capacities as judged by the increase in the number of viable melanocytes after 21 days of cell culture (28.2% and 26.9%, p<0.001). Concomitantly, the same ASA concentration resulted in significant decrease in the concentrations of MDA in the cell culture medium of the normal and active vitiligo melanocytes 6 hour and 21-day period following the ASA treatment [6 hour: 16.2% (p<0.05) and 18.4% (p<0.001); 21 day: 32% and 38.6% (p<0.001)]. However, the long-term (21 days) treatment of cultured melanocytes from the normal and active vitiligo donors with ASA at 300µg/ml resulted in a significant reduction in the number of viable melanocytes (33.6% and 63.5%, p<0.001). Whereas, MDA concentrations 6 hour and 21-day period following the ASA treatment had significantly increased [6 hour: 28.6% (p<0.05) and 41.3% (p<0.001) 21 day: 92.8% and 127.8% (p<0.001)].

Conclusion: Low-dose ASA (75µg/ml) may confer protection of skin melanocytes from the normal and active vitiligo donors against lipid peroxidation and up-regulate their proliferative capacities. On the other hand, high-dose ASA (300µg/ml) may have deleterious effects on the melanocytes, increasing lipid peroxidation and hence may potentiate melanocyte apoptosis.


Melanocytes are one of 3 major populations of cells in the human epidermis, which include the Langerhan cells and keratinocytes. The primary function of the melanocyte is the synthesis of an inert molecule, melanin in response to all types of inflammatory stimuli that generate radical oxygen in
the skin. Moreover, melanocytes are capable of antigen presentation; hence, they can participate in normal skin immunity. Functional epidermal melanocytes disappear from involved skin in patients with vitiligo. The root cause of vitiligo remains unresolved, however, several hypotheses or mechanisms have been proposed to explain the etiology of it that involve intrinsic melanocyte abnormalities, specific anti-melanocyte antibodies and cytotoxic damage to melanocytes. Nevertheless, none proved fully explanatory or satisfactory. Recently, an oxidative stress has been suggested to be the initial pathogenic event leading to melanocyte degeneration or apoptosis. Evidence has been presented for hydrogen peroxide (H$_2$O$_2$) accumulation and defective recycling of 6-tetrahydrobiopterin (6BH$_4$), the cofactor of phenylalanine hydroxylase (PAH) in the entire epidermis of patients with active vitiligo. Consequently, this results in the buildup of the epidermal L-phenylalanine and the isoform of 6BH$_4$, 7BH$_4$ with decreased PAH and 4a-hydroxy-LBH$_4$ dehydratase activities that collectively lead to further generation of H$_2$O$_2$. Several other impaired metabolic steps have been identified in vitiliginous epidermis that yield H$_2$O$_2$. FT-Raman spectrometric measurements on vitiligo skin revealed high concentrations of H$_2$O$_2$. Accumulation of H$_2$O$_2$ can deactivate key enzymes in the epidermis. Low catalase and glutathione peroxidase levels have been demonstrated in patients with active vitiligo. Vacuolation and lipid peroxidation in epidermal cells of vitiligo skin biopsies have also been described. The melanocyte proliferation can be regulated by various skin and systemic growth factors and cytokines. In the lesional area of vitiligo skin, some of these cytokines are imbalanced. A significantly lower expression of basic fibroblast growth factor (bFGF) and stem cell factor, and a significantly higher expression of the inflammatory cytokines, interleukin–6 and tumor necrosis factor-α (TNF-α) were detected compared to non-lesional and healthy skin. Furthermore, vitiliginous epidermis was found to have prominent infiltrate of T-cells and the up-regulation of intercellular adhesion molecule-1 (ICAM-1) and the human lymphocyte antigen molecules. Hence, active vitiligo may be considered as an inflammatory disease. The arachidonate cascade includes the cyclooxygenase (COX) pathway to form prostanooids and the lipooxygenase (LOX) pathway to generate several oxygenated fatty acids, the eicosanoids that may play a dual role in regulating cells survival and apoptosis through unknown mechanism. Leukotrienes (LTs) are products of the 5-LOX pathway of arachidonic acid metabolism. They can be divided into 2 general classes by structure and activity. Leukotriene B$_4$ (LTB$_4$) is a 5,12-dihydroxyeicosatetraenoic acid that is profoundly chemotactic to human polymorphonuclear leukocyte. It is also known to be a major product of activated neutrophils and macrophages. The cysteinyl leukotrienes (LTC$_4$, LTD$_4$ and LTE$_4$) comprise the second class. LTC$_4$ and LTD$_4$ but not LTB$_4$ or LTE$_4$, have been shown to be potent mitogens for cultured human neonatal melanocytes. Moreover, LTB$_4$ and LTD$_4$ are important signals for melanocytes pigmentation, thus, LTC$_4$ was shown to stimulate and maintain long term growth of cultured adult human melanocytes, whereas LTB$_4$ stimulate human melanocyte pigmentation. Leukotrienes are present in elevated amounts in various inflammatory dermatoses and post-inflammatory hyperpigmentation. Recently, it was shown that melanocyte is capable of synthesizing its own LTs as demonstrated by its release of LTB$_4$ and LTC$_4$. In active vitiligo, LTC$_4$ release rate from cultured melanocytes was significantly reduced, whereas that of LTB$_4$ was significantly increased. ASA (aspirin) causes an irreversible inhibition of the constitutive COX-1 and down-regulation of the COX-2 pathway. Accordingly, the aim of the present study is to investigate the effects of ASA at 2 different concentrations on the proliferation and lipid peroxidation of cultured melanocytes obtained from skin of uninvolved skin of patients with active vitiligo.

Methods. The following investigations were carried out from February 2001 through to November 2001, at the Vitiligo Unit, King Abdulaziz University Medical Center, Jeddah, Kingdom of Saudi Arabia.
**Melanocyte culture.** Primary skin cultured melanocytes that were previously cryopreserved in liquid nitrogen, were thawed out at room temperature. These melanocytes were originally cultured from skin biopsy (2 cm²) of 7 patients with active vitiligo of different types and duration and 7 age matched normal healthy individuals with no known family history of vitiligo serving as control as described previously. The activity of vitiligo was based on the vitiligo disease activity score (VIDA-score) represented by a 6-point scale. Appearance of new macules or progression and enlargement of depigmented lesions with no spontaneous repigmentation were taken as proof of vitiligo activity. The melanocytes were suspended and washed in a keratinocyte serum free medium (KSFM) (Gibco, Grand Island, New York, United States of America) of high calcium concentration (2 mM Ca++) as described previously. They were then transferred into T-25 canted neck flask containing 5 ml KSFM supplemented with human recombinant bFGF (10ng/ml), endothelin-1 (10nM), genetin (25µg/ml), penicillin (100U/ml), and streptomycin (100µg/ml). The melanocytes were kept in a humidified incubator with 5% CO₂ at 37°C and allowed to grow to confluence with the medium being changed once every 3 days.

**Effects of ASA on the proliferation and lipid peroxidation of cultured melanocytes.** The half-maximal inhibitory concentration (IC₅₀) for ASA on COX-1 activity in cell culture is 0.3 ± 0.2 µg/ml and for COX-2 is 50 ± 10 µg/ml. Therefore, 2 concentrations of ASA were chosen to investigate their effects on melanocytes proliferation and lipid peroxidation. The first one (75µg/ml) represents slightly higher concentration than the IC₅₀ for COX-2, and the other one exceeds the IC₅₀ value by at least a factor of 5. The sub-cultured melanocytes in the second passage (approximately 1.5 x 10⁶ cells) of the normal individuals and patients with active vitiligo were seeded in T-25 canted neck flasks, and allowed to grow for 21 days in the supplemented KSFM containing the 2 different concentrations of ASA (75 and 300µg/ml), with one flask serving as a control, and the medium was changed once every 3 days. At the end of the 21-day period, the melanocytes were detached using Sigma's cell detachment solution (Sigma-Aldrich), incubated at 95ºC for one hour, and then cooled on ice for 5 minutes. Butan-1-ol (5ml) was then added. All tubes were vortex mixed and centrifuged at 15,000 x g for 10 minutes. The upper layer was removed, and the MDA-specific fluorescence was measured at 552 nm using a scanning luminescence spectrometer. The detached melanocytes were analyzed using Sigma's Cel Lytic B solution, and the protein concentration was determined by Bradford technique using a commercially available kit (Bio-Rad, Richmond, California, United States of America), and counted using the Coulter counter, model ZM.

Malondialdehyde (MDA) is an end product derived from the peroxidation of polyunsaturated fatty acids and related esters. Therefore, its assay can be used as a measure of lipid peroxidation in human cells. Since MDA exists as the water soluble enolate anion at physiological pH, much of the MDA generated from lipid peroxidation in cell culture may be in the culture medium. Incubation of cells with serum-free medium such as KSFM induces lipid peroxidation, and it has been shown that approximately 95% of the total MDA is released into the culture medium within 5 hours. Moreover, the use of this system which does not include the use of insulin prevents interference in the lipid peroxidation assay. Therefore, prior to melanocytes detachment, the KSFM was aspirated, centrifuged at 450 x g for 5 minutes to remove cell debris, and the resulting clear supernatant was processed for MDA assay. The assay was carried out according to the method described previously. Briefly, the sample volume of culture supernatant (2ml) was mixed with 2-thiobarbituric acid (Sigma-Aldrich), incubated at 95°C for one hour, and then cooled on ice for 5 minutes. Butan-1-ol (5ml) was then added. All tubes were vortex mixed and centrifuged at 15,000 x g for 10 minutes. The upper layer was removed, and the MDA-specific fluorescence was measured at 552 nm using a scanning luminescence spectrometer. The detached melanocytes were analyzed using Sigma's Cel Lytic B solution, and the protein concentration was determined by Bradford technique using a commercially available kit (Bio-Rad, Richmond, California, United States of America), and counted using the Coulter counter, model ZM.

**Results.** The clinical and demographic data of the patients with active vitiligo (n=7) from which the skin biopsies were originally obtained and the primary and secondary melanocytes were cultured, are shown in Table 1. The average age of the vitiligo patients is 27.6 years (range 18-42 years), whereas for the normal control is 27.7 years (range 18-38 years). The average duration of the disease is 5.3 years (range 2-13 years). It is noted that 3 of the patients had strong family history of the disease, with the presence of vitiligo in the first degree relatives (father, mother, brother(s) or sister(s)). These patients could remember vividly either a physical trauma or psychological turmoil prior to the onset of vitiligo. Two patients showed weak family history of vitiligo in which distant relatives had vitiligo.
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Table 1 - The clinical and demographic data of patients with active vitiligo.

<table>
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<tr>
<th>Patients</th>
<th>Age</th>
<th>Gender</th>
<th>Type of vitiligo</th>
<th>VIDAScore</th>
<th>Duration (Yrs)</th>
<th>Family history</th>
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<td>5</td>
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<td>+++</td>
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<td>Generalized</td>
<td>+4</td>
<td>13</td>
<td>+++</td>
<td>Car accident</td>
</tr>
</tbody>
</table>

+++ strong, + weak, VIDA - vitiligo disease activity stage, Yrs - years

Effects of ASA on the proliferation and lipid peroxidation of cultured melanocytes. Cell proliferation at 21 day period. With respect to control (ASA-untreated melanocytes), treatment of the cultured melanocytes of the normal and active vitiligo donors (n=7) with 75µg/ml ASA resulted in a significant percentage increase in the proliferation of melanocytes (28.2% and 26.9% p<0.001) (Figure 1).

However, treatment with 300µg/ml ASA, resulted in a significant percentage decrease in the proliferation of melanocytes of normal and active vitiligo donors (33.6% and 63.5%, p<0.001) (Figure 1).

Malondialdehyde concentrations in cell culture medium at: Six-hour period. The MDA concentration (measured in n mol/mg total protein) in the culture medium of the ASA-untreated melanocytes of active vitiligo donors was significantly higher than that of the normal (5.30 ± 0.39 and 6.67 ± 0.28, p<0.05), a percentage increase of 25.8% (Figure 2). Treatment of cultured melanocytes of the normal and active vitiligo donors with 75µg/ml concentration of ASA resulted in significant decrease in the MDA concentrations (4.44 ± 0.21 (p<0.05) and 5.44 ± 0.20 (p<0.001), a percentage decrease of 16.2% and 18.4% (Figure 2). However, treatment with 300µg/ml ASA resulted in significant decrease in the MDA concentrations (3.76 ± 0.18 and 4.49 ± 0.23 (p<0.001), a percentage decrease of 32% and 38.6%, (Figure 2). However, treatment with 300µg/ml ASA resulted in significant increase in the MDA concentrations in cell culture media of the normal and active vitiligo melanocytes (10.66 ± 0.63 and 16.63 ± 0.61, p<0.001), a percentage increase of 92.8% and 127.8% (Figure 2).

Discussion. Although the in vitro and in vivo studies on various metabolic mechanisms have elucidated the important pathophysiological events leading to melanocytes disappearance in vitiligo, however, the precise step in the cascade for initiation of the disease process remains obscure.35-39 Recent studies have confirmed that an imbalance of the intracellular redox status and the increased production of epidermal H2O2 may constitute important factors in the pathogenesis of melanocyte apoptosis in vitiligo.40-42 However, the proposed intrinsic oxidative stress may not and could not be looked at as the root cause of melanocyte death, rather reduced levels of the melanocyte antioxidant component(s), namely, the intracellular reduced glutathione (GSH) might be the dormant causative factor. Such reduced levels of those antioxidant component(s) may not be considered adequate or efficient enough in counteracting the increased oxidative stress triggered by various mechanisms such as trauma, emotional stress, or viral infection, hence leading to further depletion of this/those already low-level antioxidant component(s). Consequently, accelerating the release of the apoptotic cytokines that may ultimately bring the destruction of melanocytes in vitiligo. It was previously reported that the decreased antioxidant capacity in aged skin might cause an increased accumulation of ROS, which effect signaling pathways and lead to skin aging.43 Intrinsic...
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oxidative stress in melanocytes from the patients with active vitiligo may initiate lipid peroxidation, an autocatalytic mechanism that lead to oxidative destruction of cellular membranes.\(^\text{24}\) Their destruction can lead to cell death and also to the production of toxic and reactive aldehyde metabolites called free radicals. MDA is the most important of these free radicals,\(^\text{45}\) and hence it is considered as a useful marker of lipid peroxidation. The measured concentrations of MDA in the culture medium of melanocytes from the normal individuals may be regarded as relatively high which may suggest that skin melanocytes produce great amount of oxidants in the form of ROS during melanin formation more than probably that of other skin cells, such as fibroblasts. This may render them more susceptible to exogenous oxidative stress. In fact, melanocyte shows unusual sensitivity to injury. The LD\(_{50}\) for peroxide mediated killing of melanocyte is 100-fold lower than fibroblast and considerably lower than keratinocyte or endothelial cell.\(^\text{46}\) That, could be one of the reasons that might explain the greater sensitivity of cultured melanocytes towards any change in the active components of the culture medium. The cultured melanocytes of the patients with active vitiligo showed even greater increase in the MDA levels than those of their normal counterpart, which may explain the reduced yield of melanocytes cultured from the normally pigmented skin of those patients\(^\text{47,48}\) and the increased sensitivity to oxidative stress.\(^\text{49}\) In a recent study, it was shown that MDA could form an adduct with various proteins, including collagen in cultured fibroblasts. These MDA-modified proteins were shown to decrease the proliferative capacities of cells, strongly altering cell cycle progression by blocking passage to G2/M phases, and induced apoptosis.\(^\text{50}\) Therefore, it is wise to search for a chemical agent that can protect the melanocytes from the deleterious consequences of oxidative stress that may cause an increase in lipid peroxidation. In the present report, ASA at 75\(\mu\)g/ml was shown to lower the MDA concentrations in the cell culture medium of the normal and active vitiligo donors, which may reflect a decrease in the process of lipid peroxidation. This effect of ASA may be explained as follows. The salicylic acid moiety of ASA at 75\(\mu\)g/ml may stimulate the biosynthesis of intracellular GSH of melanocytes, hence increasing its antioxidant status thereby ameliorating the oxidative stress. This is may be supported by the finding that a serum salicylate level of 0.52mmol/l in rabbit produce a 97% increase in glutathione levels in the liver cells as well as significant inhibition of lipid peroxidation.\(^\text{51}\) Also, aspirin per se was found to be a strong antioxidant. Aspirin was reported to be an efficient hydroxyl radical scavenger and an inhibitor of silica-induced lipid peroxidation, DNA strand breakage, nuclear factor-kappa B (NF-\(\kappa\) B) activation, and TNF-\(\alpha\) production.\(^\text{52,53}\) Furthermore, aspirin at 1 \(\mu\)M concentration was found to reduce both O\(^2-\) production and lipid peroxidation significantly, in rat brain homogenate.\(^\text{54}\) Hence, it might be said that ASA at 75\(\mu\)g/ml could confer melanocyte protection against melanogenic cytotoxicity produced during melanin synthesis or induced by specific exogenous oxidants. The increased proliferative capacities of cultured melanocytes from both normal and vitiligo donors in the presence of ASA (75\(\mu\)g/ml) may be attributed to the antioxidant properties of ASA, as well as its inhibitory effect on the activity of the inflammatory enzyme, COX-2. There is a strong evidence...
sugests that active vitiligo may be regarded as an inflammatory skin disease. Several reports have shown that increased ROS production during aging might enhance the expression COX-2 mRNA. Therefore, ASA (75µg/ml) treatment of cultured melanocytes may cause an inhibition of COX-2, that may constitute the predominant isoform in active vitiligo, hence may down-regulate the formation of the inflammatory proteases, prostaglandins (PGE3 and PGE12), mediators and cytokines that can down-regulate the proliferative capacities of the cultured melanocytes. Moreover, the release rate of the strong melanocyte mitogen, LTC4 was found to be increased significantly from cultured melanocytes of active vitiligo patients following ASA (75µg/ml) treatment. All of these changes associated with ASA treatment, collectively may cause an improvement in the microenvironmental conditions that may favor the increased proliferation of the skin cultured melanocytes. On the other hand, treatment with 300µg/ml ASA of the cultured melanocytes from the normal and active vitiligo donors produced significant increase in lipid peroxidation and a decrease in the number of viable melanocytes. The effect is obviously much greater on the melanoenst of active vitiligo donors, which could be due to the greater sensitivity of these cells to chemical injury. It may be postulated that ASA at 300µg/ml may act as an oxidizing agent depleting the melanocytes from its essential antioxidant component(s) rendering it more susceptible to both intrinsic and extrinsic oxidative stress. In fact, it was demonstrated that high dose aspirin exerts significant toxicity to guinea pig myocardium and human erythrocytes, resulting in the impairment of the antioxidant system and an increase in lipid peroxidation. High dose ASA may cause excessive release of O2 from mitochondria. A recent study demonstrated that salicylate at 1 M in cultured hepatocytes lowers the threshold for the onset of the mitochondrial permeability transition (MPT), hence potentiating both necrotic and apoptotic cell.

In conclusion, ASA at 75µg/ml may confer protection of cultured melanocytes of normal and active vitiligo donors against lipid peroxidation and up-regulate their proliferative capacities. Conversely, ASA at 300µg/ml may cause a dramatic increase in melanocyte lipid peroxidation that may potentiate melanocyte apoptosis, especially those obtained from patients with active vitiligo. The clinical importance of these results is that low dose (300mg) aspirin may be orally administered to vitiligo patients in the hope to halt the activity of the disease. Moreover, stimulating the proliferative capacities of remaining melanocytes, hence may cause an induction of repigmentation. The increased lipid peroxidation of melanocytes from patients with active vitiligo may make these cells more susceptible to apoptosis if they were to be exposed to oxidants or oxidative stress. This may explain the increased disease activity following frequent exposure to sun light.

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References


