The effect of acetylsalicylic acid on the release rates of leukotrienes B_4 and C_4 from cultured skin melanocytes of active vitiligo

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ABSTRACT

Objective: The aim of this study is to investigate the effect of the non-steroidal anti-inflammatory agent, acetylsalicylic acid (ASA), otherwise known as aspirin, at different concentrations on the release rates of the pro-inflammatory mediators, leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) from in vitro cultured melanocytes obtained from normal pigmented skin of patients with active vitiligo.

Methods: This study was carried out between April, 2000 and September, 2001, at The Vitiligo Unit, King Abdul-Aziz University Medical Center, Jeddah, Kingdom of Saudi Arabia. Skin biopsies were obtained from patients with active vitiligo (n=7) of different extent and duration, and normal healthy age-matched individuals (n=7) serving as control were recruited to the study. The release rates of LTB_4 and LTC_4 were determined before and after the addition of the ASA at 3 different concentrations (15, 75, 150 µg/ml) in the primary skin melanocytes culture medium using a commercially available kit based on radioimmunoassay method.

Results: Following the ASA treatment at 3 different concentrations (15, 75 and 150 µg/ml), the release rates of LTB_4 and LTC_4 were increased from melanocytes of the normal individuals (13%, 7.5% and 30%; 7.2%, 51.4% and 60.7%, $p<0.001$). However, in patients with active vitiligo, the release rate of LTB_4 from melanocytes was decreased (2.9%, 14.4% and 7.4%, $p<0.05$), whereas that of LTC_4 was increased (3.9%, 93.8% and 101.4%, $p<0.001$).

Conclusion: Acetylsalicylic acid at therapeutic concentrations can regulate the release rates of LTB_4 and LTC_4 from cultured skin melanocytes of normal and active vitiligo subjects.

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Vitiligo is a common skin disorder that is characterized by the appearance of amelanotic macules, which generally begins at puberty and tends to progress over time. The prevalence of vitiligo is estimated to approximately 1% of the world population regardless of race, ethnic background, or gender.[1] The disease may cause serious psychological problems for those afflicted.[2] Several in situ microscopical investigations, performed with a number of melanocytes antigens, showed that melanocytes are absent from vitiliginous skin,[3,4] strongly supporting the notion of melanocytes destruction rather than melanocytes dedifferentiation into immature melanocyte precursors as proposed by other investigators.[5] The loss of melanocytes from the vitiliginous epidermis may cause profound changes in the cutaneous immunity, since it is, recently, recognized that melanocytes participate in normal skin physiological immune system.[6] Moreover, melanocytes obtained from hypopigmented skin at the margin of lesional area and from normal

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appearing skin of vitiligo patients exhibited evidence of damage such as, dilated rough endoplasmic reticulum.[7] The etiology of vitiligo remains obscure, however, several theories have been proposed to explain its pathogenesis. The most popular are the cytotoxic,[8,9] autoimmune,[10-12] and neural[13,14] hypotheses. A composite or convergence theory has been suggested, combining all of the 3 hypotheses of vitiligo.[15] Recently, a group of investigators has proposed that vitiligo is a disease of entire epidermis involving an overproduction and accumulation of the epidermal phenylalanine hydroxylase cofactor, 6-tetrahydrobiopterin ($6BH_4$) and its 7-isomer ($7\text{-}BH_4$).[16] Exposure to ultraviolet radiation can cause photolytic oxidation of $6BH_4$ and sepiopterin with concomitant release of hydrogen peroxide (H_2O_2) in the epidermis.[17] Moreover, $6BH_4$ can react with molecular oxygen leading to the formation of more H_2O_2,[18] hence explaining the accumulated levels of epidermal H_2O_2 in these patients. Hydrogen peroxide can also efficiently oxidize $6BH_4$ as well as $7BH_4$ to 6- and 7- biopterin.[19] 6-Biopterin, in micromolar amounts can be very cytotoxic to melanocytes.[17] In association with the high levels of H_2O_2 in the vitiliginous epidermis, catalase activity was shown to be very low.[20] Therefore, a sudden burst of H_2O_2 can be very cytotoxic and may explain active depigmentation. Another group of biological modifiers known to be participants in the immune-inflammatory process are the arachidonic acid metabolites: the prostaglandins and leukotrienes. The melanogenic effect of ultraviolet B on the murine epidermis can be abrogated by administration of indomethacin to irradiated mice.[21] Consequently, it was suggested that prostaglandins might be involved in the stimulation of melanin synthesis. The role of leukotrienes namely leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) has not been thoroughly investigated as pro-inflammatory mediators in regulating melanocyte growth. To our knowledge, the available data that elucidate the role of melanocyte as an autocrine regulator of its own growth is scarce. It was demonstrated that LTC_4 and its metabolites LTD_4 and LTE_2 exhibited unique mitogenic effect on cultured human melanocytes.[22] Leukotriene C_4 was also found to stimulate melanocyte chemokinesis and chemotaxis in vitro.[23] In a recent published data it was demonstrated that the release rate of LTC_4 was substantially decreased from cultured skin melanocytes of patients with active vitiligo,[24] while that of LTB_4 was markedly increased.[25] It was suggested then, that this imbalance in the release rates of LTB_4 and LTC_4 might have been due to an imbalance in the redox status of these melanocytes, which may arise primarily from the intrinsic low antioxidant capacity, namely the intracellular reduced glutathione (GSH). Acetylsalicylic acid (ASA) (aspirin) and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the biosynthetic activity of the 2 isoforms of the cyclooxygenase (COX-1 and COX-2).[26] The inhibition by aspirin is due to the irreversible acetylation of the COX site of prostaglandin H_2 synthase, leaving the peroxidase activity of the enzyme unaffected. The inhibition by ASA of either of the 2 isoforms of COX induces an activation of the 5-lipooxygenase (5-LOX) with an increased synthesis of leukotrienes. Moreover, it was reported that aspirin may possess radical scavenging properties and hence may confer protection of cells against the deleterious effects of H_2O_2 and superoxide anions.[27-28] Accordingly, the aim of the present study is to investigate the effect of ASA on the release rates of LTB_4 and LTC_4 from cultured melanocytes obtained from normal pigmented skin of patients with active vitiligo.

Methods. Seven patients with active vitiligo of different types and duration, and 7 healthy age-matched individuals with no known family history of vitiligo serving as control were selected for this study. This study was carried out at the Vitiligo Unit, King Abdul-Aziz University Medical Center, Jeddah, Kingdom of Saudi Arabia, between April 2000 to September 2001. The control were 5 males aged 18, 20, 26, 32 and 38 years and 2 females aged 32 and 28 years. The activity of vitiligo was based on the vitiligo disease activity score (VIDA-score) represented by a 6-point scale.[29] The selected patients had VIDA-score of +4 and +3 (active in the past 6 weeks and 3 months) and skin photo type 4 or 5. Written consent was taken from each donor in compliance with guidelines set by the ethical committee of King Abdul-Aziz University Medical Center. The patients were requested to stop all forms of vitiligo medical treatment for at least 2 weeks prior to skin biopsy procedure.

Skin biopsy. Shaved skin biopsies of approximately 1 cm^2 were obtained from the forearm of the normal donors and from the uninvolved skin of active vitiligo patients at different sites and 5-10 cm away from a lesional area. The biopsy site was anesthetized with 1% xylocaine. The skin biopsy was immediately placed in a freshly prepared keratinocyte serum free medium (KSFM) (Gibco, Grand Island, NY, USA) containing the necessary antibiotics and fungizone.

Melanocyte culture. The fresh skin biopsies were placed in 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution for 2 hours at 37˚C to separate the epidermal layer. The trypsin-EDTA solution was replaced by KSFM and the epidermal layer was gently vortex mixed for 30 seconds to free the epidermal cells from the stratum corneum. The method of Horikawa et al[30] was employed to

establish the primary melanocytes culture. Briefly, the cells were seeded in 25-cm^2 T-flasks containing 10 ml KSFM supplemented with 10 ng/ml human recombinant basic fibroblast growth factor (hrb FGF), 10 nM endothelin-1, and bovine pituitary extract (Gibco), in the presence of calcium (0.09 mM) and 25 µg/ml geneticin (G418). After the total elimination of fibroblasts and keratinocytes from the culture medium, the calcium concentration was raised to 2 mM. Subsequently, melanocytes were allowed to grow to confluence for 28 days, with a medium change once every 3 days.

Determination of the release rates of LTB$_4$ and LTC$_4$ from melanocytes following ASA treatment. An approximately 0.5 million of the primary cultured melanocytes were seeded into 25-cm^2 T-flask containing 10 ml of supplemented KSFM and incubated with 2 µCi ^{3}H-arachidonic acid (specific activity = 83.8 Ci/m Mol; New England Nuclear, Boston, MA, USA) for 24 hours as previously described.[31] Then, the KSFM was aspirated and the melanocytes were washed 3 times with Kreb-Henseleit solution, and seeded into 6-well culture plate (5-6 x 10^4 cells/well) containing the supplemented KSFM with added ASA at 3 different concentrations (15, 75 and 150 µg/ml). The melanocytes were, then, incubated for 2 hours, after that aliquots of 0.5 ml of the culture medium from each well were transferred into sterile conical polystyrene tubes and stored at -70ºC until analysis.

Measurement of LTB$_4$ and LTC$_4$ was performed using LTB$_4$ and LTC$_4$ radioimmunoassay kits according to the manufacturer instructions (Seragen Inc., Boston, MA, USA). The test was carried out in duplicates. The average values were expressed as picograms released per million cells per hour and plotted in histogram forms.

Statistical analysis. The data obtained were analyzed statistically using the Statistical Package for Social Sciences 10.0 computer program package. Results were expressed as means ± SEM and comparison between the means of the release rates of leukotrienes from melanocytes in 3 concentrations of ASA were compared using the analysis of variance (ANOVA) test. *p* value of <0.05 were considered significant.

Results. **Table 1** shows the clinical and demographic data of 7 patients with active vitiligo. The average age of those patients was 27.6 years (range 18-42 years), whereas for the normal control was 27.7 years (range 18-38 years). The average duration of the disease was 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. The primary harvest of melanocytes obtained from the patients with active vitiligo was less than that obtained from the normal individuals.

The release rates of LTB$_4$ and LTC$_4$ from melanocytes of normal and active vitiligo donors following ASA treatment. The release rate (measured in pg/1 million cells hour) of LTB$_4$ from the cultured melanocytes of the normal individuals (n=7) was 17.55 ± 0.80 (control), and was significantly increased in the presence of ASA (15,

Table 1 - The clinical and demographic data of patients with active vitiligo.

Patients	Age	Gender	Type of vitiligo	VIDA-score	Duration (years)	Family history	Remarks
1	29	Female	Generalized (acrofacial)	+3	5	+++	Physical trauma
2	25	Female	Localized (arms and thighs)	+3	3	+++	Marriage tension
3	29	Female	Localized (chest)	+4	3	-	Depression
4	19	Female	Generalized (mixed)	+3	7	+	-
5	31	Male	Generalized (vulgaris)	+4	4	+	-
6	18	Male	Localized (acrofacial)	+3	2	-	-
7	42	Male	Generalized (vulgaris)	+4	13	+++	Car accident

+++ = strong, + = weak, VIDA-score - vitiligo disease activity score

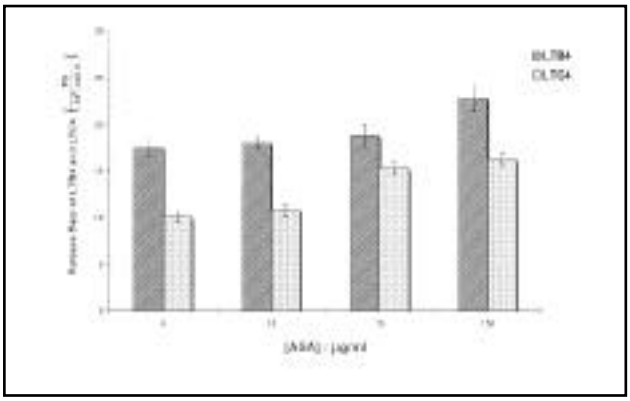

Figure 1 - The release rates of leukotriene B_4 (LTB_4) from cultured skin melanocytes of the healthy normal subjects and patients with active vitiligo (n=7) following acetylsalicylic acid (ASA) (15, 75 and 150 µg/ml) treatments. Columns and vertical bars represent means ± SEM. $p<0.05$, LTC_4 - leukotriene C_4

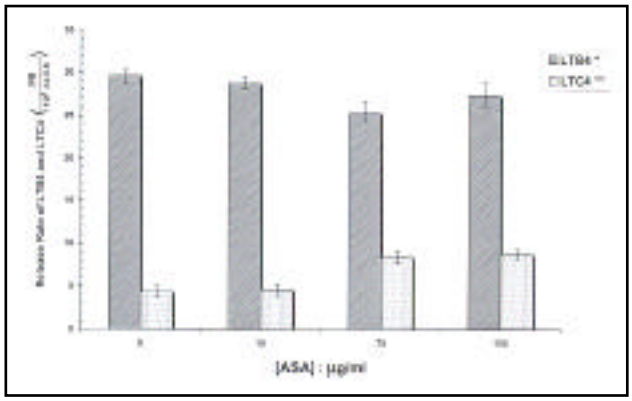

Figure 2 - The release rates of leukotriene C_4 (LTC_4) from cultured melanocytes of the healthy normal subjects and patients with active vitiligo. (n=7) following acetylsalicylic acid (ASA) (15, 75 and 150 µg/ml) treatments. Columns and vertical bars represent means ± SEM. $p<0.001$, LTB_4 - leukotriene B_4

75 and 150 µg/ml) as follow: 19.84 ± 1.30 (13%), 18.85 ± 1.22 (7.5%) and 22.81 ± 1.39 (30%), respectively, $p<0.001$. Similarly, the release rate of LTC_4 was 10.13 ± 0.59 (control), and was significantly increased as follow: 10.86 ± 0.68 (7.2%), 15.34 ± 0.74 (51.4%) and 16.26 ± 0.58 (60.7%), respectively, $p<0.001$ (**Figures 1 & 2**).

The release rate of LTB_4 from cultured melanocytes of the patients with active vitiligo (n=7) was 29.6 ± 1.09 (control), and was significantly decreased using the 3 concentrations of ASA, thus 28.39 ± 1.7 (2.9%), 25.34 ± 0.84 (14.4%) and 27.39 ± 1.7 (7.4%), respectively, $p<0.05$. However, LTC_4 release rate was 4.34 ± 0.24 (control), and was significantly increased as follow: 4.51 ± 0.24 (3.9%), 8.41 ± 0.56 (93.8%) and 8.74 ± 0.63 (101.4%), respectively, $p<0.001$ (**Figures 1 & 2**).

Discussion. There is compelling evidence suggests that active vitiligo may be categorized as an inflammatory disease, as demonstrated by the presence of mononuclear infiltrate around the dermoepidermal junction in patients with generalized vitiligo.[32] Melanocytes and neural cells are both derived from the ectoderm during embryonic development and hence it is not surprising that they have many structural and biochemical features in common. In human brain tissue, the COX-1 and its isoform COX-2 which catalyze the first 2 steps in the biosynthesis of prostaglandins from the substrate, arachidonic acid (AA) were expressed constitutively as evidenced by the presence of equal amounts of messenger ribonucleic acid for both enzymes.[33] The most interesting feature of COX-2 in the central nervous system is that it is up-regulated by normal or by abnormal nerve activity.[34] Accordingly, it may be argued that melanocyte, under normal conditions may express both COX-1 and COX-2 constitutively. Recently, significant cytokines changes were demonstrated in vitiliginous skin, including higher expression of the tumor necrosis factor – α (TNF-α) than in normal skin.[35] It is also well-established that TNF-α is one of the key cytokines that initiate and stimulate the activation of the nuclear factor-kappa B (NF-κB), possibly through an increased in its gene expression.[36] The NF-κB pathway was shown to be involved in the pathogenesis of the inflammatory response;[37] hence, a further evidence for the inflammatory nature of active vitiligo. Consequently, COX-2 may be up-regulated in the active phase of vitiligo making it, possibly, the predominant COX isoform. The fact that the half-maximal inhibitory concentrations (IC_{50}) for ASA on COX-1 activities were 0.3 ± 0.2 and for sodium salicylate were 35 ± 11 while on COX-2 activities were 50 ± 10 µg/ml and sodium salicylate were 100 ± 16 µg/ml,[38] suggests that there are may be 2 mechanisms involved in the inhibition of COX-1 and COX-2. The first is the irreversible acetylation of COX-1 at low concentrations of acetyl group or the presence of salicylate at high concentrations. The second is the inhibition of COX-2 by either acetyl group or salicylate at high concentrations. Inhibition of COX-1 or COX-2 by ASA may lead to the accumulation of AA, the primary substrate for both the COX and 5-LOX pathways. Consequently, the 5-LOX pathway may further be stimulated to overproduce the different types of leukotrienes including LTB_4 and LTC_4, hence, an increased rate of their release from melanocytes. In accordance, the data presented in this study demonstrated the increased release rate of LTB_4 and LTC_4 from the cultured melanocytes of the normal individuals following ASA treatment.

Leukotriene B_4 is an end-product of one of the 3 branches of the 5-LOX system, in which LTA_4 is readily converted to LTB_4, a reaction catalyzed by LTA_4 hydrolase. Hence, LTB_4 biosynthesis may be directly related to the concentration of its primary substrate, the AA. However, LTC_4 biosynthesis depends not only on the AA levels but also on the availability of the intracellular GSH, necessary for the activity of glutathione-s-transferase, the enzyme that catalyze the conversion of LTA_4 to LTC_4. In normal melanocytes, GSH levels may be considered normal; hence, normal cellular redox state is said to exist. However, in melanocytes from the patients with active vitiligo GSH may be present at low concentrations and most of the AA may be directed to the synthesis of LTB_4 and little to LTC_4; hence explaining the high LTB_4 and low LTC_4 rates of release from these melanocytes. The salicylate moiety of ASA may induce de novo biosynthesis of GSH, by unknown mechanism, therefore, increasing the rate synthesis of LTC_4 which may explain the increased rate of its release from the melanocytes even at low concentrations of ASA (15 µg/ml). This is supported by the finding that a serum salicylate level of 0.52 m mol/l in rabbit produced a 97% increase in GSH levels in the liver cells as well as significant inhibition of lipid peroxidation.[39] In contrast, the release rate of LTB_4 from cultured melanocytes of active vitiligo patients was significantly reduced following the ASA treatment, with the highest percentage decrease at 75 µg/ml and the lowest at 15 µg/ml, which suggests that COX-2 may be the predominant isoform of the COX pathway, hence supporting the notion that active vitiligo is an inflammatory disease. The decrease in LTB_4 release rate may be attributed to the relief of the LTC_4 biosynthetic pathway, which depends on the presence of sufficient amount of intracellular GSH, its levels may be decreased in melanocytes of active vitiligo patients due to oxidative stress. It could be postulated that ASA at 150 µg/ml may cause less stimulation of melanocyte GSH than the 75 µg/ml concentration. Thus, the reduction in the observed release rate of LTB_4 was less than that produced by ASA at 75 µg/ml. Alternatively, 150 µg/ml concentration of ASA may concomitantly cause a rise in melanocyte oxidants that may reduce the levels of the proposed stimulated GSH. This was evident by the doubled increase in the release rate of LTC_4 using 150 and 75 µg/ml of ASA. This may be due to the stimulatory effect of salicylate on the intracellular biosynthesis of GSH and/or the inhibitory effect of salicylate on TNF-α expression;[37] thus, reducing the oxidative stress in melanocyte. It may be postulated that ASA at 15 µg/ml may cause an increase in the intracellular levels of GSH, however, most of this GSH may be used preferentially to encounter the oxidative stress in melanocyte, hence, the small percentage increase in the release rate of LTC_4. In a separate set of experiments using other COX-1 and COX-2 inhibitors on cultured melanocytes from patients with active vitiligo, it was shown that those agents could cause an increase in the release rate of LTB_4 while that of LTC_4 remained unchanged (Zailaie, unpublished data), which strongly indicate that ASA can act both as an anti-inflammatory agent and an antioxidant.

In conclusion, ASA at 75 and 150 µg/ml can significantly regulate the release rates of LTB_4 and LTC_4 from cultured skin melanocytes of normal individuals and patients with active vitiligo. Leukotriene C_4 is a strong melanocyte mitogen while LTB_4 may be responsible for the down-regulation of melanocyte proliferation as demonstrated previously.[24,25] The clinical utility of the results obtained may be the use of oral aspirin at a dose, that can slow down the progression of vitiligo and probably stimulate repigmentation, but at the same time does not cause the undesirable side effects that are associated with oral aspirin.

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