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Review

Melanocortin-5 receptor and sebogenesis

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ABSTRACT

The melanocortins (α-MSH, β-MSH, γ-MSH, and ACTH) bind to the melanocortin receptors and signal through increases in cyclic adenosine monophosphate to induce biological effects. The melanocortin MC_5 and MC_1 receptors are expressed in human sebaceous glands, which produce sebum, a lipid mixture of squalene, wax esters, triglycerides, cholesterol esters, and free fatty acids that is secreted onto the skin. Excessive sebum production is one of the major factors in the pathogenesis of acne. The expression of melanocortin MC_5 receptor has been associated with sebocyte differentiation and sebum production. Sebaceous lipids are down-regulated in melanocortin MC_5 receptor-deficient mice, consistent with the observation that α-MSH acts as a sebotropic hormone in rodents. These findings, which suggest that melanocortins stimulate sebaceous lipid production through the MC_5 receptor, led to our search for MC_5 receptor antagonists as potential sebum-suppressive agents. As predicted, an antagonist was shown to inhibit sebocyte differentiation in vitro, and to reduce sebum production in human skin transplanted onto immunodeficient mice. The melanocortin MC_5 receptor antagonists may prove to be clinically useful for the treatment of sebaceous disorders with excessive sebum production, such as acne.

Contents

1. Introduction . . . 202
2. Melanocortins and their receptors in skin . . . 203
3. Melanocortin MC_5 receptor and sebaceous lipid production . . . 203
4. Development of experimental models to study melanocortin MC_5 receptor and the regulation of sebum production . . . 203
   4.1. Stimulation of sebaceous lipid production in cultured human sebocytes . . . 203
      4.1.1. Melanocortins . . . 204
      4.1.2. Cholera toxin . . . 204
   4.2. Human skin transplanted onto severe combined immunodeficient mice . . . 204
5. Development of a melanocortin MC_5 receptor antagonist for sebum inhibition . . . 205
   5.1. Rationale . . . 205
   5.2. Pharmacology . . . 205
   5.3. Inhibition of sebaceous lipids in vitro and in vivo . . . 205
6. Conclusion . . . 205
Conflict of interest . . . 205
References . . . 205

1. Introduction

Sebaceous glands are microscopic, multi-lobular glandular structures within the pilosebaceous units of the skin. They produce sebum, a complex mixture of lipids including triglycerides, wax esters, squalene, cholesterol esters, and free fatty acids. The presence of wax esters and squalene is unique to the sebaceous glands (Smith and Thiboutot, 2008; Pappas, 2009). Under physiological conditions, sebaceous lipids contribute to the integrity of the skin, affect inflammatory processes, transport antioxidants to the skin surface, and have innate antimicrobial activity (Zouboulis, 2004; Kurokawa et al., 2009). In acne vulgaris, a chronic and recurring skin disorder

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affecting millions of people worldwide (Thiboutot et al., 2009), excessive sebum secretion is one of the major contributors to the pathogenesis. Interestingly, sebum composition is highly variable between species and acne is an exclusively human disease (Smith and Thiboutot, 2008). Sebaceous gland activity is hormonally regulated and the onset of acne appears at the activation of androgen production in puberty (Zouboulis and Degitz, 2004). In addition, the neuropeptides substance-P, corticotropin-releasing hormone and proopiomelanocortin-derived peptides, as well as insulin-like growth factor-1, and indirectly estrogens, play important roles in the control of sebaceous gland activity (Kurokawa et al., 2009; Makrantonaki et al., 2008; Smith and Thiboutot, 2008).

2. Melanocortins and their receptors in skin

The melanocortins, their receptors, and their different physiological activities have been studied for decades and are reviewed in other chapters of this volume. The expression of melanocortin MC_1, MC_2, MC_4 and MC_5 receptors, but not of MC_3 receptor, has been reported in human skin. Melanocortin MC_1 receptor plays an important role in skin homeostasis, including the regulation of skin and hair pigmentation (Bohm et al., 2006; Abdel-Malek et al., 1999, 2008; Slominski et al., 2005), immune and inflammatory responses (Brzoska et al., 2008), and extracellular matrix interactions (Bohm et al., 2004). Melanocortin MC_1 receptor immunoreactivity was detected in hair follicle epithelia, melanocytes, some periadnexal mesenchymal cells, keratinocytes, dermal fibroblasts, sebaceous cells, adipocytes, mast cells, and secretory and ductal epithelia of sweat glands (Bohm et al., 2002, 2006). In human sebaceous glands, melanocortin MC_1 receptor expression was observed in both undifferentiated and differentiated sebocytes (Zhang et al., 2006). The immunoreactivity of MC_1 receptor was found higher in sebaceous glands of acne patients, as compared to normal skins (Ganceviciene et al., 2009). Melanocortin MC_2 receptor has been suggested to be involved in the stress response of human skin (Slominski et al., 2000), and it may regulate lipolysis in adipocytes (Boston and Cone, 1996; Xue et al., 1998). MC_2 receptor mRNA was detected in human skin (Slominski et al., 1996), and its protein was found in normal keratinocytes (Moustafa et al., 2002). Melanocortin MC_4 receptor signaling contributes to the regulation of pigmentation in cultured cells (Spencer and Schallreuter, 2009), but its function in human skin in vivo has not been demonstrated. MC_4 receptor was detected in dermal papillae cells (Bohm and Luger, 2004) and in human epidermis, in both keratinocytes and melanocytes (Spencer and Schallreuter, 2009). The experimental inactivation of melanocortin MC_5 receptor in mice led to a phenotype of defective water repulsion, resulting from reduced sebaceous lipid production (Chen et al., 1997; Cone, 2006). In human skin, melanocortin MC_5 receptor immunoreactivity was localized to sebaceous glands, eccrine glands and hair follicles, and epidermis (Thiboutot et al., 2000). In the sebaceous gland, melanocortin MC_5 receptor is expressed only in the differentiated cells at the center of the glands, while the basal, undifferentiated cells do not express this receptor (Zhang et al., 2006). This review will focus on the role of melanocortin MC_5 receptor in the regulation of human sebaceous cell differentiation and sebum production.

3. Melanocortin MC_5 receptor and sebaceous lipid production

In rodents, melanocortin MC_5 receptor is expressed in multiple exocrine tissues (Chen et al., 1997; van der Kraan et al., 1998). Abundant expression was observed in the secretory epithelia of lacrimal, Harderian and preputial glands, but there was no detection in the ducts and stromal parts of the glands. A functional link of melanocortin MC_5 receptor to sebogenesis was first demonstrated by the phenotype of the melanocortin MC_5 receptor-deficient mouse (Chen et al., 1997). The lack of expression of this receptor led to reduced production and secretion of sebaceous lipids, such as sterol esters, resulting in severe defects in water repulsion and thermoregulation (Chen et al., 1997). Earlier studies of ablation of the neurointermediate lobe of the pituitary (the source of circulating α-MSH), found a decrease in sebaceous lipid production, (Thody and Shuster, 1975). In hypophysectomized and castrated rats, the administration of α-MSH and testosterone restored sebum secretion (Thody et al., 1976). These studies concluded that α-MSH is a true "sebotrophin" (Thody and Shuster, 1975), which is in agreement with the later melanocortin MC_5 receptor knockout study. Moreover, proopiomelanocortin-deficient mice were shown to have a similar lipid-related phenotype as the MC_5 receptor deficient mice (Yaswen et al., 1999), further suggesting that melanocortins regulate sebum production via MC_5 receptor.

4. Development of experimental models to study melanocortin MC_5 receptor and the regulation of sebum production

4.1. Stimulation of sebaceous lipid production in cultured human sebocytes

The development of tissue culture systems provides a unique tool to study sebaceous gland activity at the molecular and cellular levels. Most of the early research of sebaceous glands was conducted in animal models (the rodent preputial gland model and the hamster ear or flank models). Cells isolated from these glands were initially used to study sebocyte function in vitro (Potter et al., 1979; Matias and Orentreich, 1983; Rosenfield, 1989). However, results from animal cells and tissues are not necessarily relevant to human sebum production. Sebum composition is species-specific, and sebum-related diseases such as acne are exclusively human (Ito et al., 1998). Consequently, many attempts were made to develop human sebocyte cultures. Karasek and Charlton (1982) reported the growth and serial cultivation of sebocytes from a dermal layer rich in sebaceous glands, which was further developed and modified by Doran et al. (1991). In addition, a human sebaceous organ culture system has been established by Kealey (1990). Although these methods led to a significant advance in the field, their use was limited by an insufficient number of cells. To overcome this problem, three immortalized sebaceous cell lines were developed in the past eleven years, i.e. SZ95 (Zouboulis et al., 1999), SEB-1(Thiboutot et al., 2003), and Seb-E6E7 (Lo et al., 2008). These cell lines have been widely used in both basic and pharmaceutical research (Zouboulis et al., 2008a,b); however, SZ95 cells do not express melanocortin MC_5 receptor (Bohm et al., 2002), and there are no reports documenting MC_5 receptor in either SEB-1 or Seb-E6E7 cells.

To establish a human primary sebaceous cell culture model, we modified the sebocyte isolation protocols of Karasek and Charlton (1982) and Doran et al. (1991). Samples of facial skin were obtained from individuals 45–60 years of age undergoing facelift surgeries, with informed consent and Institutional Review Board approval. These individuals were healthy, with no history of treatments with retinoids, lasers, or chemotherapeutic agents. The skins were obtained within 5 h from the completion of surgery, and the second 0.4 mm dermal section, previously shown to be rich in sebaceous glands, was used for isolation of sebaceous cells (Karasek and Charlton, 1982; Doran et al., 1991). Following tissue digestion, the released cells were seeded on a feeder layer of mitomycin C-inactivated 3T3 fibroblasts, and were cultured using a three-stage sebocyte culture medium system (Zhang et al., 2003, 2006). In this method, the sebocytes could be induced to differentiate by agents such as the melanocortins or cholera toxin. The culture medium of the first stage contains both growth factors and serum, to stimulate proliferation. Cells were cultured next with reduced serum, followed by a third stage of serum-free medium, to induce cell differentiation and sebaceous lipid production. Low-level induction of differentiation was observed in

the serum-free medium stage, while minimal or no spontaneous differentiation was observed in serum-containing medium. Sebocyte differentiation could be enhanced by melanocortins or cholera toxin. In this system, undifferentiated sebocytes exhibited morphology similar to human epithelial cells, while the cytoplasm of differentiated cells was filled with numerous and prominent lipid droplets.

The characterization of the cultured primary sebocytes combined lipid analysis and gene expression profiling. To analyze the lipid contents of the differentiated sebocytes, extracts of [^{14}C] acetate–labeled cells were subjected to high performance thin layer chromatography analysis (Pappas et al., 2002). This analysis demonstrated that these cultured sebocytes produce squalene, triglycerides, cholesterol esters, and free fatty acids. Unlike sebaceous glands in vivo, the cultured sebocytes did not produce detectable wax esters, which may be due to the lack of fatty alcohols, necessary for wax ester synthesis. Alternatively, wax esters could not be visualized due to incomplete separation from the cholesterol esters on the high performance thin layer chromatography plate. Using quantitative polymerase chain reaction, melanocortin MC_5 and MC_1 receptors, but not MC_2, MC_3 and MC_4 receptors, were detected in the human sebocyte cultures.

4.1.1. Melanocortins

To study the role of melanocortins in the regulation of human sebaceous lipid production and sebocyte differentiation, primary sebocytes were induced by NDP-α-MSH, ACTH, or bovine pituitary extract, which contains multiple proopiomelanocortin peptides. These treatments induced the appearance of cytoplasmic lipid droplets, analyzed by high performance thin layer chromatography to contain the human sebum-specific lipid squalene. Reverse transcription polymerase chain reaction showed very low level of melanocortin MC_5 receptor expression under serum-free condition, but a strong increase following treatment with NDP-α-MSH or bovine pituitary extract (Zhang et al., 2003). Both squalene production and melanocortin MC_5 receptor induction were associated with sebocyte differentiation, suggesting an active role for melanocortin MC_5 receptor in sebaceous lipid production in this culture system. In contrast, the level of melanocortin MC_1 receptor expression was not associated with sebaceous cell differentiation, suggesting that this receptor is independent of the sebaceous lipid production process.

4.1.2. Cholera toxin

Melanocortins initiate intracellular signaling by binding to and activating G-protein coupled melanocortin receptors, resulting in an increase in cyclic adenosine monophosphate production. Cholera toxin irreversibly activates G_S protein, which in turn stimulates cyclic adenosine monophosphate production by activating adenylyl cyclase. Cholera toxin was required for sebocyte differentiation in response to specific peroxisome proliferator-activated receptor-α and -γ agonists in a rat preputial primary sebocyte culture and these agonists were ineffective without cholera toxin (Rosenfield et al., 2002).

Human primary sebocyte culture treated with cholera toxin showed profound differentiation and lipid production, as visualized by oil red O staining. High performance thin layer chromatography analysis demonstrated the production of human, sebum specific lipids, specifically squalene. Melanocortin MC_5 receptor mRNA and protein were detected only at the onset of differentiation and in fully differentiated cells, as shown by reverse transcription polymerase chain reaction and immunohistochemistry, respectively (Zhang et al., 2006). We found that NDP-α-MSH increased cyclic adenosine monophosphate production in sebocyte cultures, and induced moderate differentiation (Huang et al., 2002), while cholera toxin induced higher levels of cyclic adenosine monophosphate production and more pronounced sebocyte differentiation (Eisinger et al., unpublished data).These findings are in agreement with Rosenfield et al. (2002) demonstrating that rat preputial sebocyte differentiation requires cyclic adenosine monophosphate generation, and that cholera toxin alone could induce differentiation of sebocytes.

4.2. Human skin transplanted onto severe combined immunodeficient mice

Multiple animal models have been developed to study the sebaceous glands, including the most commonly used rat and mouse preputial gland models (Potter et al., 1979) and the hamster ear or flank models (Plewig and Luderschmidt, 1977). The glands in these models are similar to human sebaceous glands, and are androgen-responsive (Matias and Orentreich, 1983; Lutsky et al., 1975). The major limitations of these models are the species-specific nature of sebaceous lipids (Nikkari, 1974), the different anatomy of the pilosebaceous unit (Plewig and Luderschmidt, 1977), and the regulation of cell differentiation and responses to various treatments, which are much different from human (Geiger, 1995). So far, no animal model has been shown predictive in the evaluation of the effects of anti-acne drugs in human (Smith and Thiboutot, 2008).

Human skin transplanted onto severe combined immunodeficient mice retains its human properties (Juhasz et al., 1993); therefore we evaluated this model system for human sebaceous gland studies. Male severe combined immunodeficient mice were used as the recipients of the human skin grafts, as androgens are critical to sebaceous cell proliferation and differentiation (Ebling et al., 1975; Thody et al., 1976; Zouboulis et al., 2007). The skin grafts were able to regenerate human sebaceous glands that were fully functional, producing human sebum components and expressing melanocortin MC_5 receptors (Eisinger et al., 2010). This model provides valuable understanding of sebaceous gland regeneration and development, and could be used for identifying biological pathways and for testing agents that potentially regulate human sebogenesis. The limitations of this experimental system include the limited supply of human facial skins, difficulties in transplantation, a 2–3-month delay until complete regeneration of human sebaceous glands is achieved, and variable responses between donors.

At 7–8 weeks post transplantation, the human sebaceous glands of the transplanted skins are fully developed (Eisinger et al., 2010), as shown by H&E staining and the expression of epithelial membrane antigen, a specific marker of differentiated human sebocytes (Latham et al., 1989). High performance thin layer chromatography analysis of extracts of [^{14}C]acetate–labeled biopsies of the grafts documented the production of human sebaceous lipids. Total sebum output evaluated by SEBUTAPE® documented similar density, distribution, and quantity of lipids as those of in normal human skin. Within three months post transplantation, the sebaceous cells were fully loaded with lipids (Eisinger et al., 2010), and expressed high levels of melanocortin MC_5 receptor (Zhang et al., 2006).

Human sebaceous glands are regulated by androgens, and sebocytes express androgen receptors (Zouboulis and Degitz, 2004; Thiboutot, 2004). The suitability of the human skin/severe combined immunodeficient mouse model for studying sebum suppression was evaluated using flutamide, a non-steroidal androgen blocker used in acne therapy (Lutsky et al., 1975; Thiboutot, 2004; Zouboulis and Degitz, 2004). Transplanted human grafts treated with flutamide (5%) showed a decrease in sebum production, as documented by SEBUTAPE®. The production of sebaceous lipids by in vitro biopsies from the grafts was evaluated by [^{14}C]acetate-labeling and high performance thin layer chromatography, and consistent inhibition of wax esters and squalene was observed. Histological evaluation documented a decrease in the size of the sebaceous glands, with reduced levels of epithelial membrane antigen (data not shown). These results are in agreement with the previously reported reduction in size of preputial glands and sebum suppression in animal models treated with flutamide (Lutsky et al., 1975). This study confirms the

usefulness of the human/severe combined immunodeficient mice system for human sebum inhibition studies.

5. Development of a melanocortin MC_5 receptor antagonist for sebum inhibition

5.1. Rationale

There is a clinical need for the treatment of sebaceous disorders characterized by increased sebum production, such as acne vulgaris and seborrheic dermatitis. The most effective treatments for acne, isotretinoin and androgen modulators, directly affect sebaceous gland differentiation (Clarke et al., 2007; Thiboutot et al., 2009; Alexis, 2008), but have several undesired side effects. The rationale for the development of melanocortin MC_5 receptor antagonists for sebum control is based on the following facts: (1) the melanocortin MC_5 receptor is expressed at high levels in human sebaceous glands and its expression is correlated to sebaceous cell differentiation (Thiboutot et al., 2000; Zhang et al., 2006); (2) sebaceous lipids were shown to be regulated by exogenous α-MSH and testosterone (Thody et al., 1976); and (3) the targeted disruption of the melanocortin MC_5 receptor in mice resulted in down-regulation of sebaceous lipids and defective exocrine gland functions (Chen et al., 1997).

5.2. Pharmacology

JNJ-10229570 (2,3-diaryl-5 amino-[1,2.4] thiadiazole, Pan and Reitz, 2002 and Pan et al., 2003) is used as an example of a melanocortin MC_5 receptor antagonist. Both a free base form (JNJ-10229570) and a bromide salt form (JNJ-7818369) of this molecule were studied with similar results, and for simplicity this review will use only the JNJ-10229570 name. This agent inhibited the binding of [^{125}I] NDP-α-MSH to melanocortin MC_1 and MC_5 receptor membranes of over-expressing cells with IC_{50}s in the range of 200–300 nM (data not shown). We next measured the ability of this molecule to inhibit α-MSH-induced cyclic adenosine monophosphate in primary sebocytes. α-MSH increased cyclic adenosine monophosphate levels and the compound suppressed this effect, demonstrating a functional antagonism of melanocortin receptor signaling (data not shown).

5.3. Inhibition of sebaceous lipids in vitro and in vivo

The effect of the melanocortin MC_5 receptor antagonist on sebocyte differentiation and lipid production was evaluated in the primary sebocyte culture in three ways. Image analysis was used to document intracellular lipid droplets. The intracellular content of all neutral lipids was quantified using the Nile Red assay (McMillian et al., 2001). Individual lipid classes (squalene, cholesterol, wax esters, and triglycerides) were determined by [^{14}C]acetate–labeling and high performance thin layer chromatography analysis (Pappas et al., 2002). Sebocytes exposed to JNJ-10229570 showed strong inhibition of lipid droplet formation, and squalene and cholesterol esters were inhibited in a dose-dependent fashion (data not shown). Human skin grafts on severe combined immunodeficient mice were topically treated with JNJ 10229570 for about 6 weeks. This treatment led to a significant decrease in surface lipids, compared to vehicle controls, as documented by SEBUTAPE®. Similarly, [^{14}C]acetate–labeled biopsies, analyzed by high performance thin layer chromatography, showed a significant reduction in squalene and wax esters. Finally, a noticeable reduction in the size of the sebaceous cells and glands was observed. Sebaceous glands exposed to JNJ-10229570 were smaller and less differentiated, and contained reduced numbers of lipid droplets, as compared to the vehicle-treated controls (data not shown). In contrast, flutamide was less effective at reducing cell size and lipid droplet size (Lutsky et al., 1975; Thiboutot, 2004; Zouboulis and Degitz, 2004). In JNJ-10229570-treated skins, the number of epithelial membrane antigen-positive cells was reduced compared to the vehicle controls, and the intensity of the staining was decreased, further suggesting that JNJ-10229570 inhibits sebocyte differentiation (data not shown).

6. Conclusion

Multiple lines of evidence suggest that melanocortins and the melanocortin MC_5 receptor are involved in sebaceous lipid regulation. α-MSH, ACTH, and bovine pituitary extract can induce sebaceous cell differentiation in vitro with concomitant induction of melanocortin MC_5 receptor expression (Zhang et al., 2003). In vivo, the expression of melanocortin MC_5 receptor was seen only in lipid-loaded, differentiated sebocytes, and not in the basal cells of the sebaceous gland, indicating the association of this receptor with sebaceous differentiation (Zhang et al., 2006). These findings are in agreement with the central role of α-MSH in sebum regulation in rats (Thody and Shuster, 1975; Thody et al., 1976), and with the decrease in sebaceous lipids observed in melanocortin MC_5 receptor-deficient mice (Chen et al., 1997).

Understanding the regulation of sebocyte differentiation and sebaceous lipid production is necessary for improving therapy of diseases associated with excessive sebum production such as acne vulgaris. The identification of the melanocortin MC_5 receptor as a marker of sebocyte differentiation suggests a novel therapeutic target for the control of sebum production. The human sebocyte culture and human skin xenograft models discussed in this review provide unique experimental systems for both basic research and the testing of pharmaceutical candidates. Using these systems, agents were identified that were able to inhibit sebocyte differentiation and sebum production, and could possibly be used in acne therapy.

Conflict of interest

All studies presented were performed at, and were funded by, the Johnson & Johnson Skin Research Center. All authors were employed by the Johnson & Johnson Skin Research Center during the time the studies were performed.

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