phosphatase activity was observed and shown to be reversible, the protein tyrosine phosphatase activity measured in MBCD-treated keratinocytes was not significantly different at any time point when compared with the activity measured in untreated cells, indicating that disorganization of lipid rafts does not cause inactivation of protein tyrosine phosphatases. Similarly, detection of protein oxidation showed that cultures subjected to H_2O_2 treatment presented important protein oxidation, which was partially protected by preincubation with NAC (Figure 2b), but in untreated and in cholesterol-depleted cultures, protein oxidation was never detected. Finally, an ultimate experiment was performed in which intracellular ROS synthesis was directly measured by a fluorescent dye. Hydrogen peroxide induced a strong intracellular ROS production during the first 20 minutes, which was clearly decreased when preincubation with NAC was performed. ROS measurements showed that keratinocytes with a perturbed lipid raft organization presented only very low ROS levels, which were even below the baseline ROS levels measured in untreated cells (Figure 2c). In other words, all these results suggest that disruption of lipid rafts is rather able to inhibit the basal ROS production, an observation that is in total accordance with recent literature showing that MBCD treatment protects yeast cells from oxidative stress (Du and Ayscough, 2009). In conclusion, contrary to the hypothesis suggested by our previous study (Mathay *et al.*, 2008), the data presented hereby clearly point out that lipid raft disruption by cholesterol depletion does not modulate the cellular redox balance toward oxidation in normal keratinocytes. To pursue the identification of mechanisms involved in keratinocytes after cholesterol depletion, we have now undertaken transcriptional profiling. This unbiased approach should help to elucidate major actors, mechanisms, and pathways involved during and after lipid raft disruption in epidermal keratinocytes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Frédéric Minner for his excellent help with statistical analysis. This work was financially supported by FRFC grant 2.4506.01 and FNRS grant 1.5.033.06F to YP. CM holds a fellowship from the ''Fonds de la Recherche Scientifique—FNRS.''

***Conny Mathay*[1] *and Yves Poumay*[1]**

[1]*Cell and Tissue Laboratory, URPHYM, University of Namur (FUNDP), Namur, Belgium. E-mail: yves.poumay@fundp.ac.be*

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Tannic Acid and Quercetin Display a Therapeutic Effect in Atopic Dermatitis via Suppression of Angiogenesis and TARC Expression in Nc/Nga Mice

Journal of Investigative Dermatology (2010) **130,** 1459–1463; doi:10.1038/jid.2009.401; published online 7 January 2010

TO THE EDITOR

The number of atopic dermatitis (AD) patients has been increasing steadily worldwide. AD is a dangerous disorder because it not only causes chronic inflammation but also leads to bacterial and viral skin infections. Although the pathogenesis of AD is not completely understood, inflammation-related angiogenesis (Groneberg *et al.*, 2005) and cytokine production associated with T helper type 2 (Th2) polarization

Abbreviations: AD, atopic dermatitis; TA, tannic acid; TARC, thymus and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin

have been reported to be key mediators of AD development (Ong *et al.*, 2002; Howell, 2007). We therefore thought that blocking these processes might be an effective therapeutic approach for the treatment of AD.

Recently, several effective therapies and agents involving polyphenolic compounds have been introduced for AD treatment (Gottlieb, 2005). The well-known polyphenolic compounds tannic acid (TA) and quercetin have a documented anti-inflammatory effect as well as a role in balancing Th1 and Th2 responses in inflammatory human dermal fibroblasts (Park *et al.*, 2006, 2009; Rogerio *et al.*, 2007). On the basis of these reports, we hypothesized that TA and quercetin are able to inhibit AD development and investigated the therapeutic effect of these compounds on AD-like skin lesions.

In AD, production of vascular endothelial growth factor (VEGF) is enhanced. VEGF acts as a proangiogenic factor and induces hyperpermeability of blood vessels, and several reports have suggested that inhibition of its production or blockade of its action may be an effective new therapeutic strategy for treating AD (Zhang *et al.*, 2006; Ip *et al.*, 2007). However, VEGF suppression by polyphenolic compounds in AD models is not yet clearly understood. Although both TA (Bawadi *et al.*, 2005; Wen *et al.*, 2008) and quercetin have been shown to reduce VEGF levels in various cancer cells (Zhong *et al.*, 2006; Luo *et al.*, 2008). However, little is known about their effect on AD-like disease pathology.

To investigate the effect of TA and quercetin on angiogenesis associated with AD, we first measured VEGF levels in the human keratinocyte cell line, HaCaT, using PCR after treatment with various doses of TA and quercetin. Because tumor necrosis factor (TNF)-α has a role in AD development (Trompezinski *et al.*, 2004) and induces VEGF production in human keratinocytes, HaCaT cells were pretreated with TNF-α for 1 hour, followed by treatment with TA and quercetin. TA and quercetin decreased the expression level of the TNF-α-induced proangiogenic factor, VEGF, in a dose-dependent manner (Figure 1a). To study the combined effect of TA (25 μM) and quercetin (5 μM), we administered the compounds either individually or in combination and determined VEGF expression level using PCR and ELISA (RayBiotech, Norcross, GA). Interestingly, treatment with both TA and quercetin showed a more powerful effect than treatment with either agent alone (Figure 1b and c). This suggests that the combination of TA and quercetin acts as a powerful VEGF suppressor in keratinocytes.

Because the expression level of VEGF correlates with the level of angiogenesis (Carmeliet and Jain, 2000), we were interested in investigating whether treatment with both TA and quercetin had a therapeutic effect and was associated with angiogenesis *in vivo*. We divided Nc/Nga mice (Biostar AD, Kobe-shi, Japan) into three groups: a normal healthy group (negative control), an AD control group (after AD induction, only phosphate-buffered saline was applied to AD skin lesions), and a TA (25 mM) and quercetin (2.5 mM) cotreatment group (after AD induction, TA and quercetin were applied to AD lesions). Induction of AD in Nc/Nga mice was accomplished by applying house dust mite allergens (Biostar AD, Japan) to the ears for 3 weeks. The effects of treatment were then assessed for 3 weeks at the site of AD induction. As shown in Figure 1d, gross clinical assessment showed that mice treated with TA and quercetin showed significant improvement in symptoms and appeared similar to the normal healthy group.

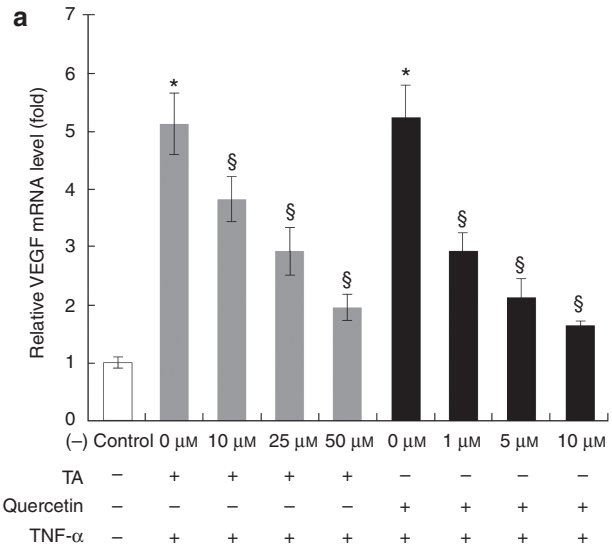
At the end of treatment, skin lesions were excised, sectioned, and stained with hematoxylin and eosin for histopathological analysis. Compared with the AD control group, the group treated with TA and quercetin showed a clear reduction in ear and epidermal thickness and decreased infiltration of leukocytes. These data suggest that the application of TA and quercetin has a therapeutic effect on AD, resulting in milder symptoms and a reduced AD score (Figure 1e).

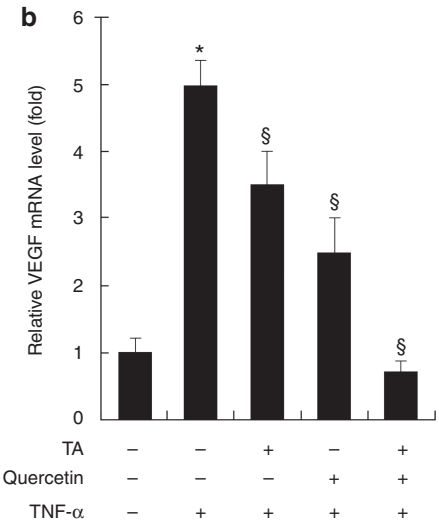
Angiogenesis level was measured using immunohistochemical staining for CD31, which is commonly used for vessel detection (Saban *et al.*, 2007). As expected, the expression of CD31 in the group treated with TA and quercetin

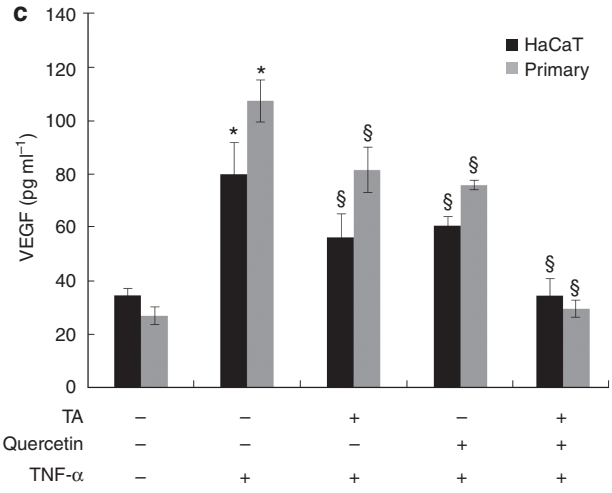
Figure 1. Therapeutic effect of tannic acid (TA) and quercetin on atopic dermatitis (AD) by suppressing angiogenesis. HaCaT cells were pretreated with or without tumor necrosis factor (TNF)-α (50 ng ml^{-1}) for 1 hour, followed by treatment with (**a**) 0, 10, 25, and 50 μM of TA and 0, 1, 5, and 10 μM of quercetin for 6 hours. (**b**) HaCaT cells were pretreated with or without TNF-α (50 ng ml^{-1}) for 1 hour, followed by treatment with 25 μM of TA and 5 μM of quercetin, separately or in combination for 6 hours. Cells were then harvested, total RNA was isolated, and cDNA was synthesized. Real-time PCR analysis was performed to detect vascular endothelial growth factor (VEGF) mRNA. Data are expressed as the ratio of VEGF to β-actin mRNA expression. (**c**) HaCaT cells and human primary keratinocytes were pretreated with or without TNF-α (50 ng ml^{-1}) for 1 hour, followed by treatment with 25 μM of TA and 5 μM of quercetin—separately or in combination—for 24 hours. The supernatant levels of VEGF were determined using human VEGF-specific ELISA kits. Results are from one representative experiment of three separate experiments. The data are reported as mean ± SD. *$P<0.05$, (−) control group versus TNF-α-treated group. §$P<0.05$, TNF-α-treated group versus group treated with TA and quercetin. (**d**) House dust mite allergens were applied to the ears of Nc/Nga mice three times a week for 3 weeks. Treatment was administered with or without TA (25 mM) and quercetin (2.5 mM) three times a week on Nc/Nga mice with induced AD, and treatment effects were assessed for 3 weeks. At the treatment end points, gross assessment (top) and histological analysis (bottom) were carried out on ear skin with induced AD. Gross view as shown by photographs and histological analysis performed with hematoxylin and eosin staining. The negative control group indicates the normal healthy group ($n=10$). The AD control group indicates the group treated with phosphate-buffered saline alone as a vehicle control after induction of AD ($n=10$). The TA-and-quercetin-treated group ($n=10$) showed significantly milder AD symptoms compared with the AD control group. The gross view and histological analysis of the TA-and-quercetin-treated group are similar to those of the negative control group. Arrows indicate the AD site. Bar = 20 μm. (**e**) At the treatment end points, the AD score of the TA-and-quercetin treatment group was lower than that of the nontreatment group (AD control). Data are reported as mean ± SD. *$P<0.05$, (−) control group versus AD control group; §$P<0.05$, AD control group versus TA-and-quercetin-treated group. (**f**) Angiogenesis levels were detected by staining CD31 (red). The expression rate of CD31 in the TA-and-quercetin-treated group ($n=10$) was significantly lower than that in the AD control group ($n=10$). The TA-and-quercetin-treated group ($n=10$) showed patterns of CD31 expression that were fairly similar to those in the negative control group ($n=10$). Bar = 50 μm.

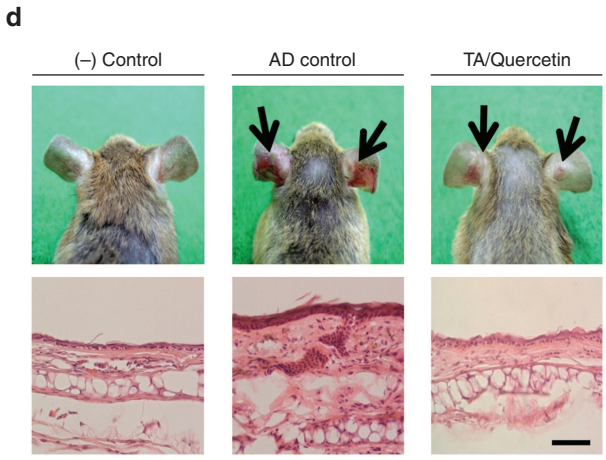
was significantly lower than that in the AD control group (Figure 1f). This is in agreement with the VEGF expression pattern *in vitro* (Figure 1a–c). These results indicate that TA and quercetin lead to the inhibition of neoangiogenesis, which may also suppress chronic inflammation in AD-like skin lesions.

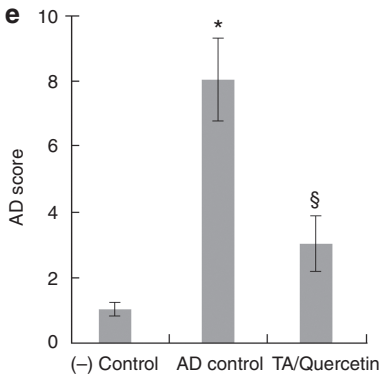
AD is characterized as an inflammatory Th2 polarity disorder (Howell *et al.*, 2007). Hence, we investigated whether the cytokine thymic stromal lymphopoietin (TSLP) correlates with the therapeutic effect of TA and quercetin in AD. TSLP induces Th2 cytokines, including thymus and activation-regulated chemokine (TARC), and is involved in allergic responses, IgE production, and eosinophilia (Liu, 2007). We measured the secretion of

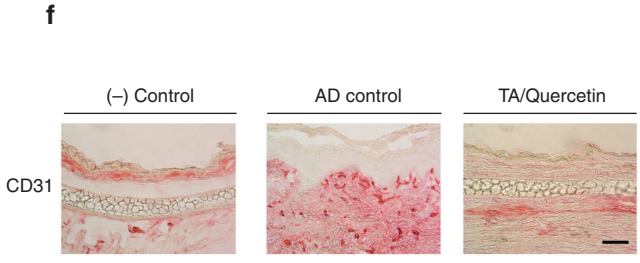












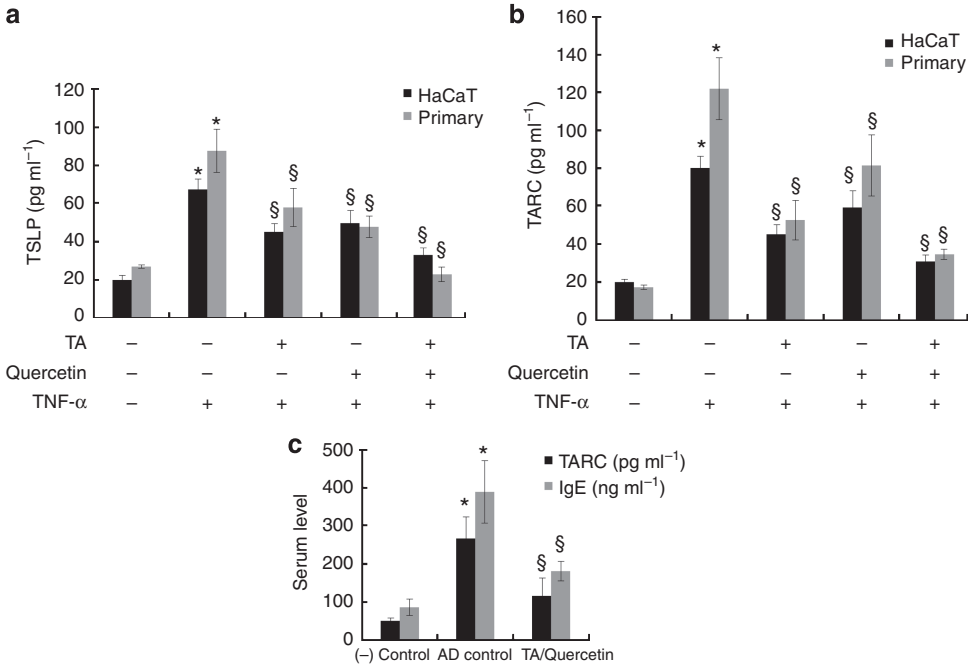


Figure 2. Inhibitory effect of tannic acid (TA) and quercetin on the production of thymic stromal lymphopoietin (TSLP), thymus and activation-regulated chemokine (TARC), and IgE. (**a**) HaCaT cells and human primary keratinocytes were pretreated with or without tumor necrosis factor (TNF)-α (50 ng ml^{-1}) for 1 hour, followed by treatment with 25 μM of TA and 5 μM of quercetin, separately or in combination for 24 hours. The supernatant levels of TSLP and (**b**) TARC were determined using ELISA kits. Results are from one representative experiment of three separate experiments. Data are reported as mean ± SD. *$P<0.05$, (−) control group versus TNF-α-treated group. §$P<0.05$, TNF-α-treated group versus TA-and-quercetin-treated group. (**c**) Treatment with or without TA (25 mM) and quercetin (2.5 mM) applied three times a week to Nc/Nga mice with induced AD was observed for 3 weeks. The serum levels of TARC and IgE were determined using specific ELISA kits. The TA-and-quercetin-treated group ($n=10$) had lower serum TARC and IgE levels compared with the AD control group ($n=10$). Results are from one representative experiment of three separate experiments. Data are reported as mean ± SD. *$P<0.05$, (−) control group versus AD control group; §$P<0.05$, AD control group versus TA-and-quercetin-treated group.

TSLP in TNF-α-stimulated human keratinocytes using ELISA (R&D Systems, Minneapolis, MN), and found that treatment with TA and quercetin suppressed TNF-α-induced TSLP expression (Figure 2a).

To further confirm that the combination of TA and quercetin regulates Th2 polarity, the TARC expression level was also measured by ELISA (R&D Systems). TARC has a role as a transporter of Th2 cells and is an important mediator of AD (Sandoval-Lopez and Teran, 2001). However, the effects of TA and quercetin on TARC production in AD-like skin lesions are not yet known. Our experiments indicate that treatment with TA and quercetin results in a marked decrease in TNF-α-induced TARC levels *in vitro* and *in vivo* (Figure 2b and c).

It is well known that an elevated level of systemic serum IgE accompanies AD-like skin lesions, and that its production is affected by TSLP and correlates with severity of AD (Liu, 2007; Morita *et al.*, 1999). Because of this, measurement of IgE is commonly used to study AD severity. In our mouse study, serum IgE was measured using ELISA (Shibayagi, Shibukawa, Japan), and we found that it was downregulated in the group receiving TA and quercetin treatment as compared with the AD control group, which is in agreement with the observation that serum IgE level corresponded with AD severity in mice with induced AD (Figure 2c). On the basis of our results, we hypothesize that TA and quercetin may decrease Th2 polarization in AD. Ultimately, this contributes to a better balance between the Th1 and Th2 pathways, and regulation of this process has a therapeutic effect on AD.

To our knowledge, this is the first report demonstrating that TA and quercetin have a therapeutic effect on AD through suppression of angiogenesis and Th2-related cytokine expression, including TSLP and TARC, in an AD-like Nc/Nga mouse model. We suggest that TA and quercetin might be an effective and improved therapeutic strategy that should be investigated further for treatment of AD. Future studies are needed to investigate whether the combination will have a therapeutic effect on AD in humans.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Korea Science & Engineering Foundation (KOSEF) through the Research Center for Women's Disease (RCWD) at Sookmyung Women's University and by the Seoul Research and Business Development Program (10582). Animal experiments were approved by the Industry–Academic Cooperation Foundation, Sookmyung Women's University (SMU-IACUC-2008-0902-001).

***Min Kyung Jung*[1], *Dae Young Hur*[2], *Seok Bean Song*[1], *Yoorim Park*[1], *Tae Sung Kim*[3], *Sa Ik Bang*[4], *Seonghan Kim*[5], *Hyun Keun Song*[2], *Hyunjeong Park*[6] and *Dae Ho Cho*[1]**

[1]*Department of Life Science, Sookmyung Women's University, Seoul, Korea;*
[2]*Department of Anatomy, Inje University*

College of Medicine, Pusan, Korea; [3]School of Life Sciences and Biotechnology, Korea University, Seoul, Korea; [4]Department of Plastic Surgery, College of Medicine, Sungkyunkwan University, Seoul, Korea; [5]Department of Pharmacology, Seoul National University College of Medicine, Seoul, Korea and [6]Department of Dermatology, St Mary's Hospital, The Catholic University, Seoul, Korea
E-mail: hjpark@catholic.ac.kr or cdhkor@sookmyung.ac.kr

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Relationship between Germline *MC1R* Variants and *BRAF*-Mutant Melanoma in a North Carolina Population-Based Study

Journal of Investigative Dermatology (2010) **130,** 1463–1465; doi:10.1038/jid.2009.410; published online 31 December 2009

TO THE EDITOR

A few previous studies have examined the relationship between germline melanocortin-1 receptor *(MC1R)* status and somatic *BRAF* mutations in melanoma. Two publications reported strong associations in three independent populations (two from Italy and one from San Francisco) (Landi *et al.*, 2006; Fargnoli *et al.*, 2008), whereas a more recent publication found no association in an Australian population-based study (Hacker *et al.*, 2010). We report our finding of no significant association between *MC1R* status and *BRAF*-mutant melanomas in a population-based study of malignant melanoma in North Carolina.

Participants in this study were 219 cases with first primary invasive cutaneous melanoma from North Carolina, one site in the population-based Genes, Environment, and Melanoma (GEM) study (Begg *et al.*, 2006). The study protocol was approved by the institutional review board of the University of North Carolina at Chapel Hill. The Declaration of Helsinki protocols were followed, and patients gave their written, informed consent. The participants were interviewed regarding their risk factors (Thomas *et al.*, 2007). The subjects were asked to have the nevi on their backs counted by a family member or friend, using a glossy colored guide to aid in differentiating between nevi and other skin lesions.

One dermatopathologist (KB) reviewed the tumors for standard histological features. The tumors were scored

Abbreviations: adj., adjusted; CI, confidence interval; CSD, chronic sun damage; MC1R, melanocortin-1 receptor; OR, odds ratio; wt, wild type