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Evidence for Microvesicle Particles in UVB-Mediated IL-8 Generation in Keratinocytes

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Abstract

Recent studies have implicated bioactive microvesicle particles (MVP) in the keratinocyte response to many environmental stressors, in partricular ultraviolet B radiation (UVB). The generation of MVP in response to UVB involves the Platelet-activating factor receptor (PAFR) and the enzyme acid sphingomyelinase (aSMase). As UVB generates some cytokines such as interleukin-8 (IL-8) in a PAFR-dependent manner, one question is if the production and release of IL-8 and MVP could be linked. Using the human keratinocyte-derived cell line HaCaT, the present *in vitro* studies indicate that pretreatment of HaCaT keratinocytes with PAFR agonist can synergize with low fluences of UVB to generate high levels of MVP as well as IL-8 protein. Treatment of cells with an aSMase pharmacologic inhibitor blocked both processes. These studies indicate the possibility that MVP could be involved in pathologic processes involving UVB-generated production of pro-inflammatory cytokines such as IL-8.

Introduction

A major question in photobiology which has yet to be sufficiently addressed is exactly how UVB, which only is absorbed appreciably by the superficial skin epidermis, can generate systemic reactions [1]. Subcellular particles released by the keratinocyte in response to environmental stressors have been implicated in this process [2,3]. Specifically, microvesicle particles (100-1000nm, MVP) which are liberated from plasma membranes, have been demonstrated to be produced from keratinocytes and human/murine skin in response to multiple bioactive effector agents including UVB and thermal burn injury [4-7]. MVP release in response to many of these effectors is via activation of the Platelet-activating factor receptor (PAFR), with the enzyme acid sphingomyelinase (aSMase) serving an essential role in this process [7]. The MVP released from keratinocytes can also carry multiple protein cytokines and the lipid mediator PAF [6,7]. Regarding the ability of MVP to carry large levels of the metabolically labile PAF, recent studies using pharmacologic/genetic blocking of the PAFR and enzyme aSMase have implicated MVP in the systemic immunosuppressive effects of UVB [7]. The glycerophosphocholine-derived mediator PAF is the most potent lipid-derived mediator as yet reported [2,3]. Regarding skin and PAF, keratinocytes both synthesize PAF and related sn-2 oxidized glycerophosphocholines (Ox-GPCs) and express functional PAFRs linked to generation of multiple protein and lipid mediators, to include PAF itself [8-10]. UVB irradiation generates both enzymatically generated PAF as well as non-enzymatically produced Ox-GPC PAFR agonists in keratinocytes [11], and multiple lines of evidence implicate PAFR activation in UVB effects. First, UVB-mediated acute inflammatory responses including pain is decreased in mice lacking PAFRs [12]. Second, studies using cells and mice sufficient/deficient in PAFRs have demonstrated that expression of many acute genes turned on by UVB are at least in part PAFR-dependent [10]. It appears that the PAF system is involved in high fluences of UVB, or in photosensitivity such as that due to loss of xeroderma pigmentosum complementary group A [13]. Finally, UVB-induced systemic immunosuppression is due to the PAFR, in particular the PAFR expressed on the skin mast cell [14-17]. In contrast, the PAFR does not appear to be involved in UVB-mediated local immunosuppression [18].

One important aspect of photobiology is the ability of the environmental pro-oxidative stressor UVB to augment the pathologic effects of other agents. In vitro studies using keratinocytes or keratinocyte-derived cell lines have reported that a numbers of biologic agents such as interleukin-1 β (IL-1β), the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and the PAF agonist carbamoyl PAF (CPAF) can synergize with UVB to generate large amounts of the potent cytokine tumor necrosis factor-α (TNF-α) [19,20]. Similarly, our group has demonstrated that CPAF can synergize with UVB in generating TNF-α in murine skin *in vivo* [20]. Yet the ability of UVB to synergize with pro-inflammatory stimuli to augment the production of other cytokines has not been explored.

UVB fluences necessary to generate MVP in keratinocytes, human skin explants, mice and human subjects appear to be excessive, at least 2-3 times the minimal erythema dose [5,7]. However, recent studies by our group have indicated that pretreatment with low-doses of CPAF or TPA can synergize with UVB in generating MVP in cells, skin explants and mice in a process involving the PAFR and aSMase [21]. Yet, the role of MVP in cytokine production is unclear. The goals of these studies were to test if UVB could synergize with CPAF to augment the production of the pro-inflammatory cytokine IL-8 and to explore the role of MVPs in this process.

Materials and Methods

Chemicals/UVB

All chemicals were obtained from Sigma-Aldrich unless indicated otherwise. UVB of cells used a Philips F20T12/UVB lamp source (Somerset, NJ, USA) using Kodacel filter to remove UVC

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(7). The intensity of the UVB source was measured before each experiment using an IL1700 radiometer and a SED240 UVB detector (International Light, Newburyport, MA, USA) at a distance of 8 cm from the UVB source.

Cell culture

The HaCaT keratinocyte-derived cell line was provided by Dr. Petra Boukamp at the German Cancer Research Center, Heidelberg, Germany [22]. HaCaT keratinocytes were grown in DMEM high glucose media with 10% FCS as previously described [4,6,7]. HaCaT keratinocytes were grown to approximately 80-90% confluence in 10 cm dishes, and washed three times with Hanks Balanced Salt Solution (HBSS) and then incubated with HBSS + 10 mg/ml fatty acid-free BSA for UVB exposures. Cells were treated with either no treatment, vehicle (0.1% Ethanol), CPAF, TPA, or the aSMase inhibitor [7,23] imipramine (50μM) post-UVB radiation. None of the chemicals used except for imipramine absorbed appreciably in the UVB spectrum.

Measurement of MVP and IL-8

MVP were isolated from cells as previously reported [5-7]. In brief, cell supernatants were collected and centrifuged at 2,000 x g for 20 minutes at 4 °C to remove cells and debris. The remaining supernatant was divided in half for MVP vs IL-8 measurements. MVP was then pelleted after 20,000 x g centrifugation at 70 minutes at 4°C from the sample supernatant. The concentrations of the MVP were determined by using a NanoSight NS300 instrument (NanoSight Ltd, Malvern Instruments, and Malvern, UK). Three 30-second videos of each sample were recorded and analyzed with NTA software version 3.0 to determine the concentration and size of measured particles with corresponding standard error. MVP generated by these model systems have been previously characterized by western blotting as expressing Annexin V with only low levels of exosome specific markers CD63 and Tsg 101, and by transmission electron microscopy which revealed MVP with appropriate dimensions [7]. IL-8 was determined via Human Il-8 ELISA kit (R & D Systems) as previously described [7]. Values were normalized to cell numbers.

Statistics

All statistical calculations were performed using Prism 6. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%, or Student t test.

Results and Discussion

The first studies tested the doseage responsiveness of IL-8 release in HaCaT epithelioid cells. HaCaT is a spontaneously immortalized (by heat and high calcium levels) cell line derived from primary human keratinocytes [22] which expresses functional PAFRs [9]. As noted in Figure 1A, treatment of HaCaT cells with the metabolically stable PAFR agonist carbamoyl PAF (CPAF) resulted in increased IL-8 release into the supernatants. Exposure of these cell types with various fluences of UVB resulted in increased levels of IL-8 release at 400 J/m^2 and above (Figure 1B).

Our next studies examined the dose-responsiveness of MVP release in HaCaT keratinocytes from the identical supernatants from Figure 1. As depicted in Figure 2, both UVB- and CPAF treatment resulted in a dose-dependent increase in MVP release into the supernatants. These findings are similar to those previously reported by our group [4,7,21]. Comparison of Figures 1 and 2 reveal overall similar patterns of release of IL-8 and MVP into supernatants.

Though UVB has been reported to generate MVP in cells, human skin explants, mice and human subjects [4-7], the fluences necessary for MVP release are high. Inasmuch as bioactive mediators such as PAF agonists, IL-1β, and TPA can augment UVB-induced production of cytokines such as TNF-α [19,20], we next tested whether these combinations could result in synergistic release of MVP and IL-8. As depicted in Figure 3, pretreatment with CPAF one hour before fluence of UVB which does not generate significant MVP resulted in high levels of these subcellular particles in HaCaT keratinocytes. Of importance, IL-8 levels were also augmented by combination of CPAF + UVB (Figure 3B).

To ascertain a potential linkage between MVP and IL-8 release, we took advantage of our previous studies indicating that MVP release in response to CPAF and UVB was dependent upon activation of the enzyme aSMase [7,23,24]. To that end, we tested the ability of treatment with the aSMase functional inhibitor imipramine following UVB to block both the MVP as well as IL-8 release. As noted in Figure

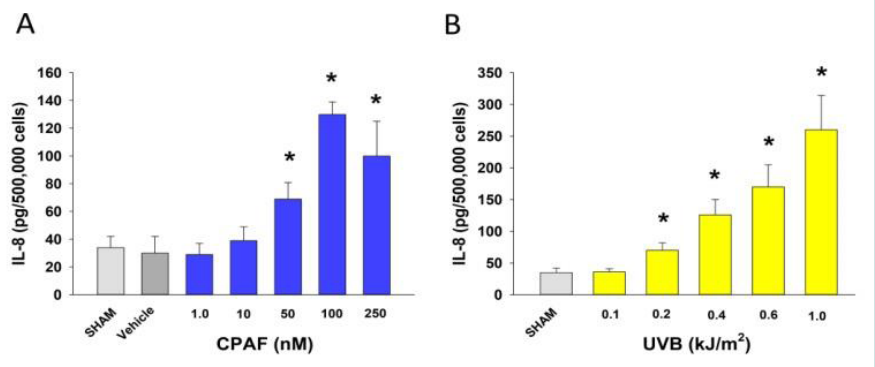

Figure 1: Dosage-responsiveness of IL-8 release by CPAF and UVB in HaCaT keratinocytes. A), B). HaCaT keratinocytes were treated with no treatment (Sham), 0.1% ethanol vehicle, or various concentrations of the PAFR agonist CPAF (A) or fluences of UVB (B) and 4 h later supernatants removed and IL-8 quantified. The data are mean ± SE IL-8 normalized to cell numbers from three separate experiments using duplicate samples. *Denotes statistically significant ($P < 0.05$) changes from sham (UVB) or vehicle (CPAF) by Student t test using Prism 6.

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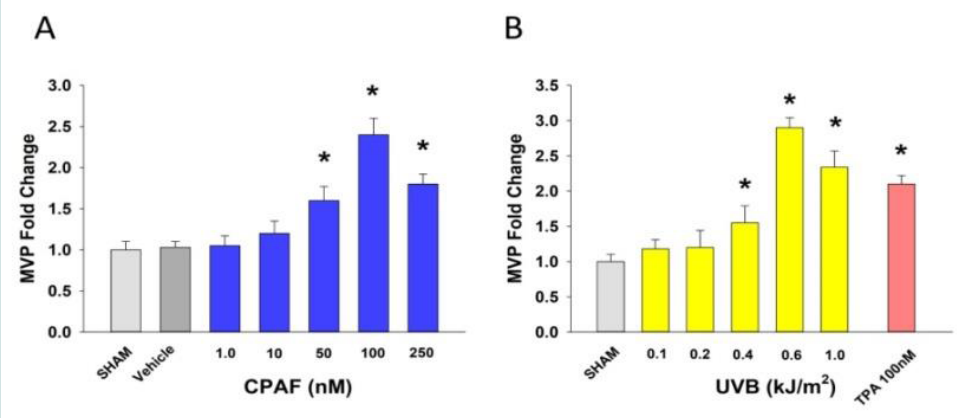

Figure 2: Dosage-responsiveness of MVP release by CPAF and UVB in HaCaT keratinocytes. A), B). HaCaT keratinocytes were treated as outlined in Figure 1 but MVP quantitated from supernatants. 100 nM of the phorbol ester TPA was used as a positive control. The data are mean ± SE MVP normalized to cell numbers from three separate experiments using duplicate samples. Baseline levels of MVP were 4.2 x 109 particles per 105 cells. *Denotes statistically significant ($P < 0.05$) changes from sham (UVB) or vehicle (CPAF/TPA) by Student t test using Prism 6.

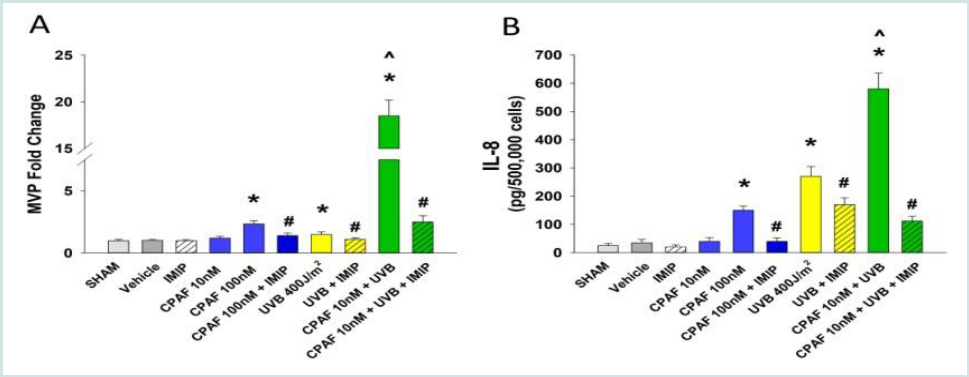

Figure 3: PAFR agonist augments the release of both MVP and IL-8 in an imipramine-sensitive manner in HaCaT keratinocytes. HaCaT keratinocytes were treated with no treatment (NT), 0.1% ethanol vehicle (VEH), or various concentrations of the PAFR agonist CPAF, or irradiated with various fluences of UVB. In some experiments CPAF was given 1 h before UVB irradiation, and 50μM imipramine (IMIP) given immediately after final CPAF/UVB. Supernatants were removed and MVP/IL-8 quantified. The data are mean ± SE fold change in MVP or concentrations of IL-8 protein normalized to cell numbers from three separate experiments using duplicate samples. *Denotes statistically significant ($P < 0.05$) changes by Student t test using Prism 6. #Denotes statistically significant ($P < 0.05$) differences induced by imipramine post-treatment compared with CPAF/UVB or the combination using Student t test using Prism 6. ^Denotes statistically significant ($P < 0.05$) differences between combination of CPAF + UVB in comparison to either CPAF or UVB alone by two-way ANOVA.

3, imipramine blocked the discharge of both MVP as well as the cytokine IL-8.

Pathologic responses from multiple agents acting in synergistic fashion are likely more common than are currently appreciated. An example of this relating to photobiology is the report of a woman who developed a severe life-threatening toxic epidermal necrolysis reaction from the self-treatment of a minor morbilliform drug eruption from ibuprofen with a single tanning bed exposure [25]. The current studies demonstrate that UVB when combined with a PAFR resulted in augmented IL-8 release, which adds to previous studies reporting synergistic TNF-α responses from UVB and other bioactive agents [19,20]. Consistent with our previously reported work examining MVP release from keratinocytes and skin [7,21], use of the aSMase inhibitor imipramine indicates this enzyme is involved in both the MVP generation as well as IL-8 release. This finding suggests that MVP and IL-8 release are linked. Moreover, aSMase inhibition could be a tool to dissect the roles of MVP in generation of other cytokines as well as in UVB pathologies.

The role of IL-8 in UVB responses is as yet unclear. However, the ability of this cytokine to activate and recruit neutrophils suggest that IL-8 could be important in acute UVB responses. A report using keratinocyte-fibroblast cocultures that treatment with a neutralizing antibody against IL-8 blocked UVB-induced production of fibroblast neprilysin and matrix metalloproteinase I suggest that UVB-induced IL-8 could have functional consequences [26].

In summary, the current studies reveal that PAFR activation can synergize with UVB resulting in augmented IL-8 release in a keratinocyte-derived cell line. The pattern of IL-8 and MVP release as well as the ability of pharmacologic inhibition of aSMase to block both processes suggests that IL-8 and MVP are related. Future studies could define further the roles of MVP and other subcellular particles such as exosomes in UVB processes including cytokine release.

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