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necrotic morphologic changes, nonapoptotic cell death and caspase-8 defects, we hypothesized that the HVJ-E–induced death of SK-N-SH cells is actually necroptosis. To confirm this hypothesis, we first used the RIP1 inhibitor necrostatin-1. The pretreatment of SK-N-SH cells with necrostatin-1 before HVJ-E exposure significantly suppressed cell death by inhibiting ROS production without inhibiting PARP cleavage (Fig. 3A–C). We confirmed that the HVJ-E–induced cell death was suppressed by pretreatment with necrostatin-1 also in SK-N-AS cells (Fig. 3C). To show that the lack of caspase-8 sensitizes the cells to necroptosis, caspase-8 cDNA was transferred to SK-N-SH cells. The restored caspase-8 in SK-N-SH cells resulted in the significant suppression of HVJ-E–induced cell death (Supplementary Fig. S2A and S2B). From these data, we suspected that HVJ-E–induced cell death might associate with the RIP1–RIP3 necrosome in caspase-8–deficient neuroblastoma cells. We used necrosulfonamide as the inhibitor of necroptosis, which blocks further downstream of necroptosis. The pretreatment of SK-N-SH cells with necrosulfonamide before HVJ-E exposure also significantly suppressed the cell death as well as necrostatin-1 (Supplementary Fig. S3). Knockdown of RIP3 using siRNA also suppressed the HVJ-E–induced cell death (Supplementary Fig. S4A and S4B). To further evaluate the involvement of RIP1 and RIP3 in HVJ-E–induced cell death, we demonstrated the HVJ-E–induced formation of RIP1–RIP3 necrosome by *in situ* proximity ligation assay, which can detect the protein–protein interactions using two antibodies derived from different species (Supplementary Fig. S5). These results imply that HVJ-E induces necroptosis in SK-N-SH cells.

HVJ-E induces cell death in neuroblastoma cells by enhancing the cytoplasmic Ca^{2+} concentration

We next attempted to unveil the upstream actions of HVJ-E–induced necroptosis in SK-N-SH cells. Necroptosis requires the phosphorylation of RIP1, which forms a complex with the TNF-α receptor in its ubiquitinated form before it is deubiquitinated by CYLD (9). By using ^{32}P labeling, we demonstrated that RIP1 phosphorylation was induced by HVJ-E treatment for 2 hours, and this phosphorylation was suppressed by RIP1 knockdown (Fig. 3D). If this

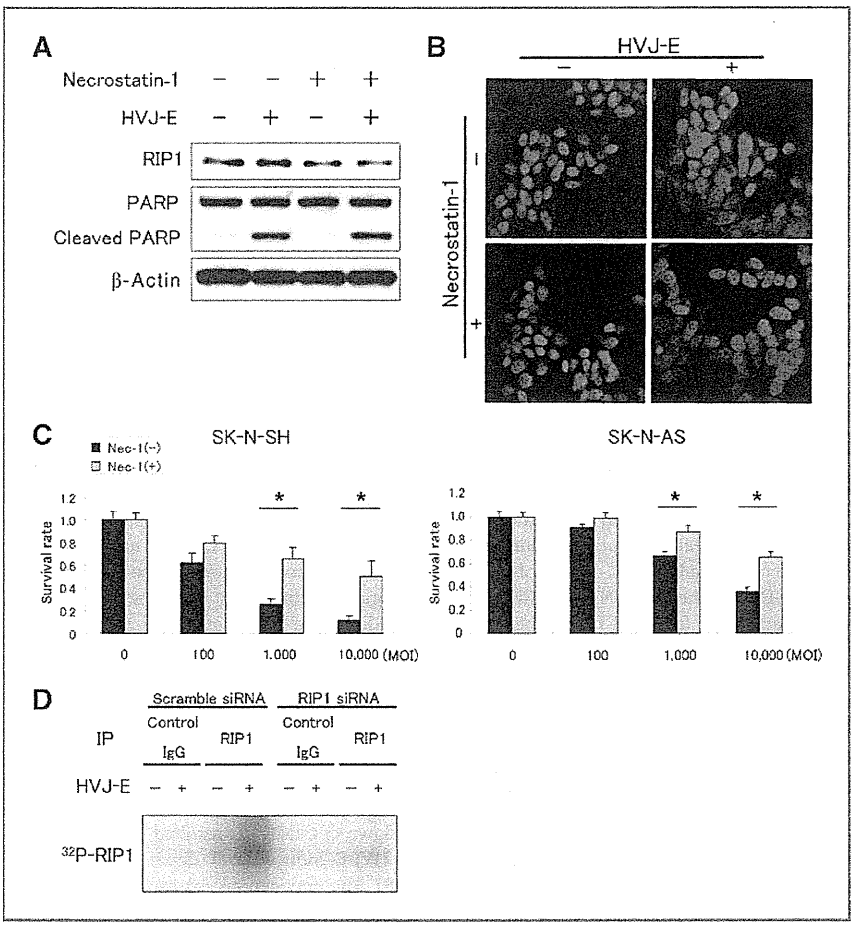

Figure 3. HVJ-E induces necroptosis in SK-N-SH cells via RIP1 phosphorylation. A, SK-N-SH cells (3×10^5 cells) with or without pretreatment with 20 μmol/L of necrostatin-1 (Nec-1) were exposed to 1,000 MOI of HVJ-E for 24 hours. Western blot analysis showed that the expression of RIP1 was slightly suppressed by pretreatment with necrostatin-1, but the cleavage of PARP was not inhibited. B, ROS production was detected by MitoSOX Red staining (red). The treatment of SK-N-SH cells with 1,000 MOI of HVJ-E induced ROS production, which was inhibited by pretreatment with necrostatin-1. The nuclei were stained with DAPI (blue). C, HVJ-E–induced cell death was significantly suppressed by pretreatment with necrostatin-1 both in SK-N-SH and SK-N-AS cells. D, SK-N-SH cells (1×10^6 cells) were transfected with RIP1 siRNA for 24 hours. Two hours after labeling of the transfected cells with 3.7 MBq of ^{32}P, the cells were treated with 1,000 MOI of HVJ-E for 2 hours and then immunoprecipitated with anti-RIP1 antibody. HVJ-E induced RIP1 phosphorylation, which was suppressed by the knockdown of RIP1. Each survival value (mean ± SD; $n = 4$) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. *, $P < 0.05$.

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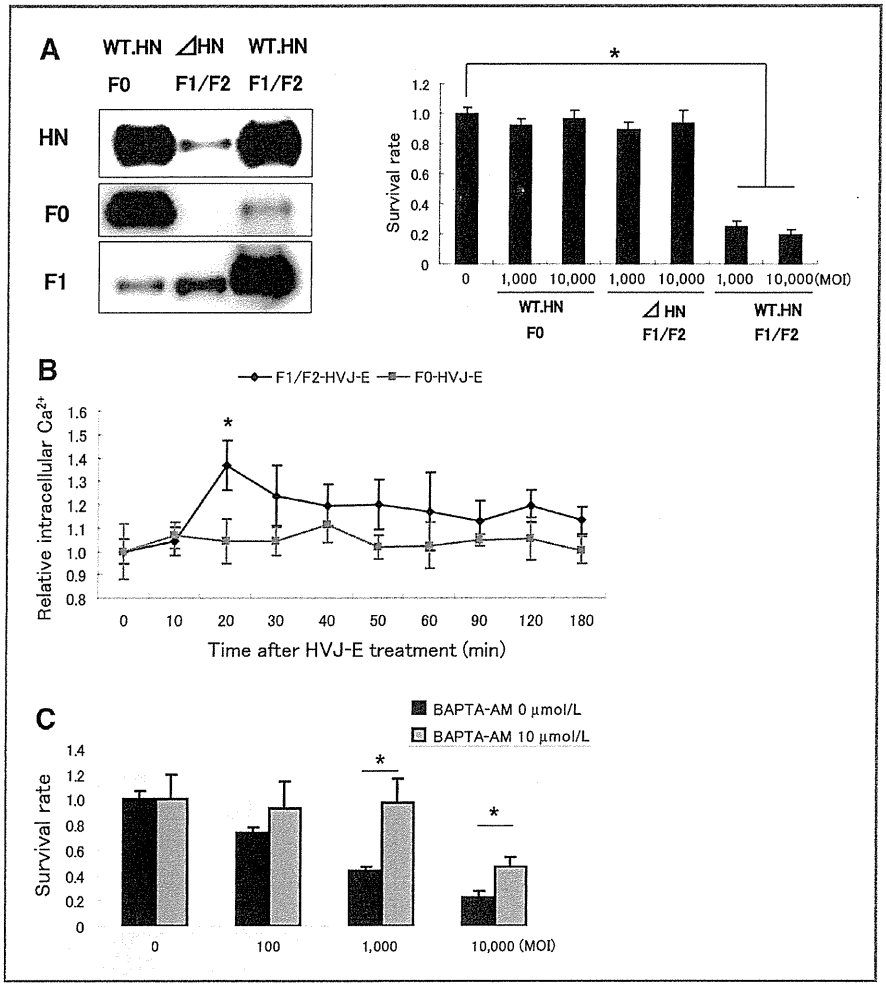

Figure 4. HVJ-E induces cell death in SK-N-SH cells by enhancing the intracellular Ca^{2+} concentration. HVJ particles produced by LLC-MK2 cells were either treated with trypsin for cleaving the F0 protein to form F1/F2 protein or heated to denature the HN protein. A, the expression of the HN, F0, or F1 proteins was detected by Western blot analysis. WT.HN, wild-type HN; ΔHN, denatured HN. Twenty-four hours after the treatment of SK-N-SH cells (3×10^5 cells) with 1,000 or 10,000 MOI of the various types of HVJ-E, only the HVJ-E with WT.HN-F1/F2 protein could kill the cancer cells. B, SK-N-SH cells (1×10^4 cells) were treated with 1,000 MOI of HVJ-E with WT.HN-F0 or WT.HN-F1/F2 protein. Only the HVJ-E with WT.HN-F1/F2 could enhance the intracellular Ca^{2+} concentration. C, HVJ-E–induced cell death was inhibited by pretreatment with BAPTA-AM. Each survival value (mean ± SD; $n = 4$) or intracellular Ca^{2+} concentration reflects the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. The experiments were performed in triplicate, and representative results are shown. *, $P < 0.05$.

SK-N-SH cell death is mediated by the TNF receptor, necroptosis should be induced by exposure to TNF-α. However, TNF-α was not able to induce cell death in SK-N-SH cells, and moreover, the TNF receptor was not endogenously expressed in SK-N-SH cells (Supplementary Fig. S6A and S6B), implying that this HVJ-E–induced cell death is not mediated by TNF receptor signaling. We confirmed that SK-N-SH cells could be killed only by HVJ-E with the WT HN protein (WT.HN) and HVJ-E with an activated fusion (F) protein (F1/F2) but not by HVJ-E with either denatured HN (ΔHN) or inactivated F (F0; Fig. 4A). Moreover, HVJ-E with F1/F2 significantly increased the cytoplasmic Ca^{2+} concentration compared with HVJ-E with F0 at 20 minutes after treatment of the SK-N-SH cells with 1,000 MOI of HVJ-E (Fig. 4B). This HVJ-E–induced cell death was significantly suppressed by pretreatment with the Ca^{2+}-chelating agent BAPTA-AM (Fig. 4C), suggesting that a cytoplasmic Ca^{2+} increase induces cell death and that this increase in Ca^{2+} requires the fusion of HVJ-E to its target cells by the activated F protein.

RIP1 phosphorylation in necroptosis is mediated by CaMK II

These results imply that a cytoplasmic Ca^{2+} increase upregulates HVJ-E–induced necroptosis. RIP1 activation requires the phosphorylation of serine 161. Among the serine/threonine kinases, protein kinase C (PKC) and CaMK II are downstream of Ca^{2+} signaling and, therefore, they may be candidates for RIP1 phosphorylation. Pretreatment with the PKC inhibitor bisindolylmaleimide before HVJ-E exposure did not significantly suppress HVJ-E–induced cell death, suggesting that PKC is not related to this type of cell death (Supplementary Fig. S7). Next, we detected the phosphorylation of CaMK II after treating SK-N-SH and SK-N-AS cells with HVJ-E (Fig. 5A). The HVJ-E–induced phosphorylation of CaMK II was inhibited by pretreatment with BAPTA-AM, implying that the increase in intracellular Ca^{2+} activates CaMK II (Fig. 5B). Knockdown of CaMK IIα resulted in the significant suppression of HVJ-E–induced cell death (Supplementary Fig. S8A and S8B). The endogenous expression of CaMK IIβ was not detected (data not shown). Moreover, the inhibition

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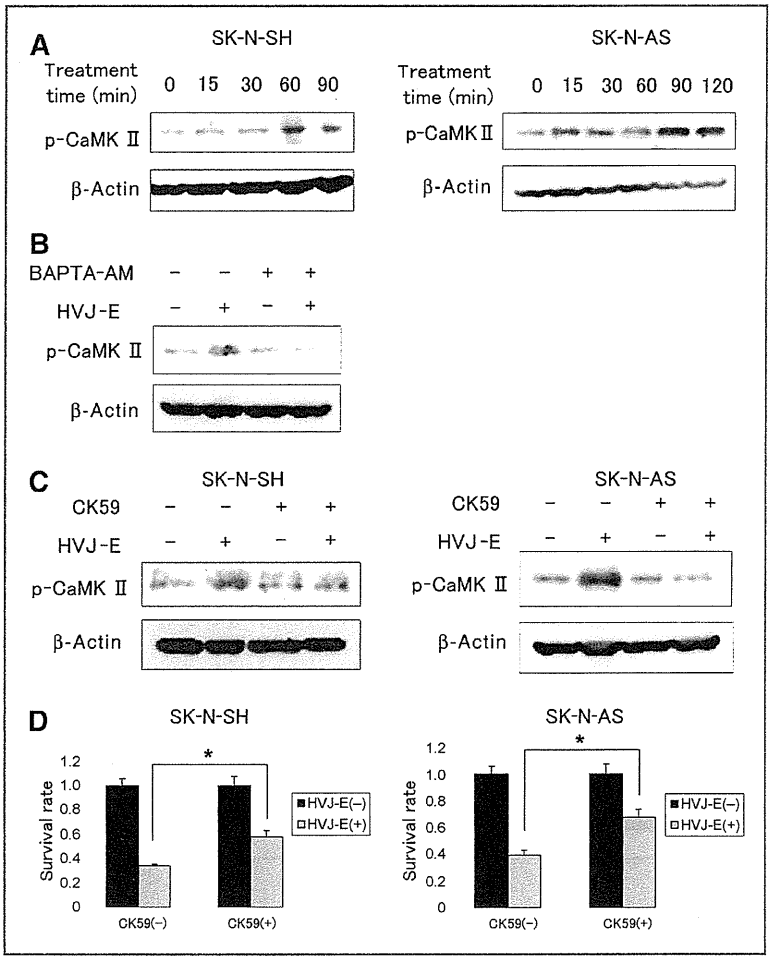

Figure 5. HVJ-E induces cell death via CaMK II phosphorylation. A, SK-N-SH and SK-N-AS cells (3×10^5 cells) were treated with 1,000 MOI of HVJ-E, and CaMK II phosphorylation was detected by Western blot analysis. B, the HVJ-E–induced phosphorylation of CaMK II was inhibited by pretreatment with BAPTA-AM in SK-N-SK cells. C, the HVJ-E–induced phosphorylation of CaMK II was inhibited by pretreatment with CK59, which was confirmed by Western blot analysis. D, the HVJ-E–induced cell death was significantly suppressed by pretreatment with CK59 both in SK-N-SH and SK-N-AS cells. Each survival value (mean ± SD; $n = 4$) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. Experiments were performed in triplicate, and representative results are shown. *, $P < 0.05$.

of CaMK II phosphorylation by pretreatment with the CaMK II inhibitor CK59 significantly suppressed HVJ-E–induced cell death in SK-N-SH and SK-N-AS cells (Fig. 5C and D), implying that CaMK II phosphorylation is related to this type of cell death.

The final objective of this study was to investigate the relationship between necroptosis and CaMK II phosphorylation. The HVJ-E–induced phosphorylation of RIP1, which was labeled using ^{32}P, was significantly suppressed by pretreatment with necrostatin-1 and CK59 (Fig. 6A), suggesting that CaMK II phosphorylation is necessary for RIP1 phosphorylation. Moreover, using 7-Amino-Actinomycin D (AAD) staining, we confirmed that HVJ-E induced necroptosis in SK-N-SH cells and showed that this necroptosis was inhibited by CK59 (Fig. 6B). The possibility that phosphorylated CaMK II is an upstream component of RIP1-dependent necroptosis was also supported by our results showing that CK59 pretreatment before HVJ-E exposure decreased ROS production and increased intracellular ATP (Supplementary Fig. S9A and S9B). This HVJ-E–induced necroptosis was also observed in xenograft tumors derived from SK-N-SH cells in SCID mice using AnnexinV-EnzoGold and 7-AAD (Supplementary Fig. S10).

In summary, we demonstrated that HVJ-E increases the cytoplasmic Ca^{2+} concentration followed by CaMK II phosphorylation, and that this activated CaMK II induces RIP1 phosphorylation and necroptosis (Fig. 7). To show that the increase of cytoplasmic Ca^{2+} induces necroptosis, we used Ca^{2+} ionophore A23187 instead of HVJ-E. As shown in Supplementary Fig. S11A, A23187 induced SK-N-SH cell death in a dose-dependent manner, and this cell death was significantly suppressed by pretreatment with the Ca^{2+} chelating agent BAPTA-AM. Using 7-AAD staining, we demonstrated that this A23187-induced cell death was necroptosis (Supplementary Fig. S11B).

Discussion

Necroptosis is inducible in many types of cells if apoptotic death signaling is inhibited by pretreatment with Z-

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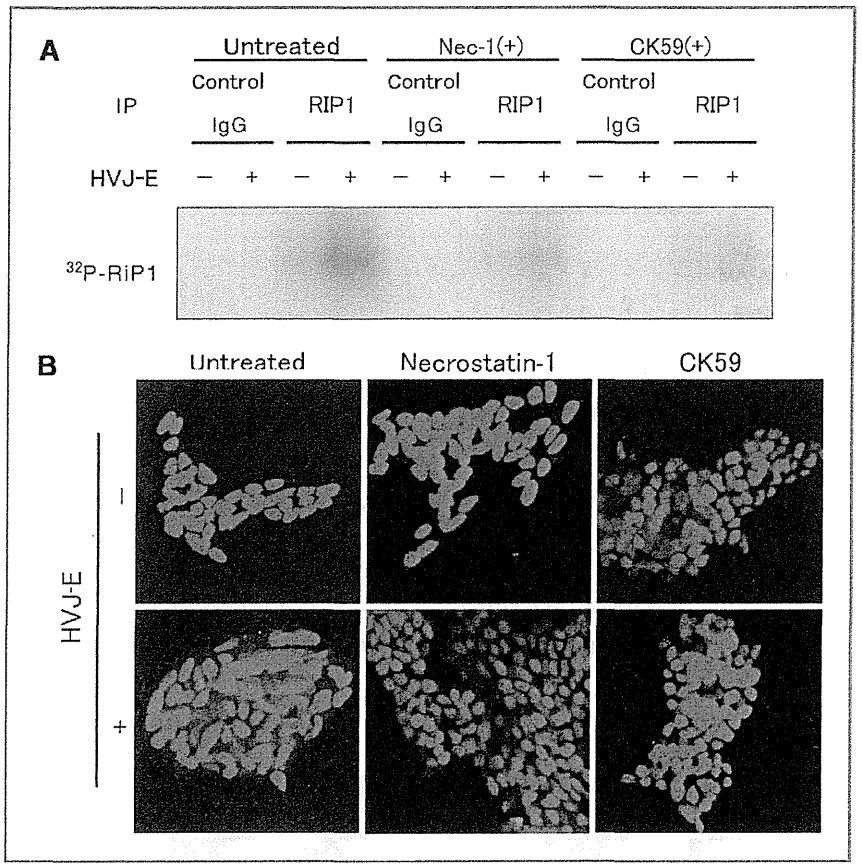

Figure 6. HVJ-E–induced necroptosis is upregulated by CaMK II. A, SK-N-SH cells (1×10^6 cells) were pretreated with necrostatin-1 or CK59. Two hours after treatment of the cells with 3.7 MBq of ^{32}P, the cells were treated with 1,000 MOI of HVJ-E for 2 hours and immunoprecipitated with anti-RIP1 antibody. The HVJ-E–induced phosphorylation of RIP1 was inhibited by pretreatment with necrostatin-1 and CK59. B, the HVJ-E–induced necroptosis, as detected by 7-AAD staining (red), was inhibited by pretreatment with necrostatin-1 and CK59. The nuclei were stained with DAPI (blue).

VAD-FMK and cycloheximide before being exposed to death ligands, such as TNF-α and the Fas ligand, and the resulting morphologic features are similar to those of necrosis (2, 3). In the ischemic brain, this type of cell death tends to be induced, and necrostatin-1 was first reported to be the agent that suppresses ischemic brain injury in mice through a mechanism that is distinct from apoptosis (16). Necrostatin-1 was later discovered to be a specific inhibitor of RIP1 (17). Therefore, cell death that is rescued by necrostatin-1 can be considered to be necroptosis, and RIP1 is believed to be the key necroptosis factor. It was recently reported that in addition to RIP1, RIP3 is essential for necroptosis because RIP3 is regulated by the caspase-8–FLIP complex (18) and mediates the embryonic lethality of caspase-8–deficient animals (19). Moreover, RIP3-knockout mice are very vulnerable to some viruses (6). Thus, necroptosis plays an important role in the inflammatory response or innate immune response to virus infection. The representative stimulator of necroptosis is TNF-α, and the mechanism of TNF-α–induced necroptosis has been well studied, but we could not study this factor here because its receptor is absent in SK-N-SH cells. Similar to HVJ-E in this study, other agents have been reported to stimulate necroptosis, such as kuguaglycoside C, a constituent of *Momordica charantia* (20). We chose RIP1 as the necroptosis marker for this study because a specific inhibitor of RIP3 was not available. The key executors of necroptosis are RIP1 and RIP3, and the downstream factors of these kinases are ROS and apoptosis-inducing factor (AIF). AIF is a Janus protein that exerts redox activity in the mitochondria and proapoptotic activity in the nucleus, but it can also regulate necroptosis (21). ROS also regulates apoptosis through other mechanisms involving AIF. Thus, the absolute markers of necroptosis that exist downstream of RIP1 and RIP3 have not yet been discovered. However, we chose ROS and ATP rather than AIF as markers of HVJ-E–induced necroptosis because a change in the oxidative phosphorylation pathway was detected in our pathway analysis. We have herein demonstrated that HVJ-E induces necroptosis in the neuroblastoma cell lines SK-N-SH and SK-N-AS.

We previously reported that HVJ-E induces apoptosis in castration-resistant human prostate cancer cells and human glioblastoma cells but does not cause toxicity in normal cells (22–24). Furthermore, we reported that HVJ-E stimulates an antitumor immune response by activating

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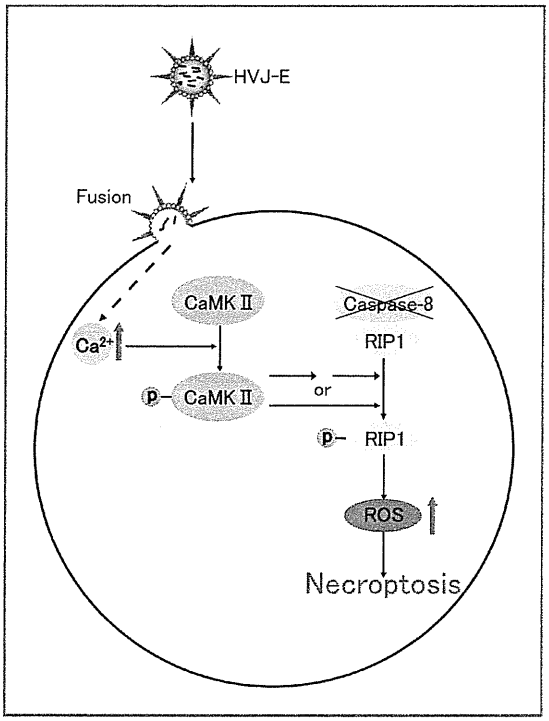

Figure 7. Schematic representation of death signaling stimulated by HVJ-E. HVJ-E enhances the cytoplasmic Ca^{2+} concentration by binding and fusing the target cells. Then, CaMK II is phosphorylated and thus activated. Once CaMK II is phosphorylated, it remains activated for a long time and exerts its kinase activity on its target proteins. Among the downstream effects of activated CaMK II, RIP1-dependent necroptosis is induced in the absence of endogenously expressed caspase-8.

cytotoxic T lymphocytes and natural killer cells and suppressing regulatory T cells (25, 26). Among these direct and indirect HVJ-E antitumor activities, we recently discovered that HVJ-E induces apoptosis via activation of the retinoic acid–inducible gene-I (RIG-I)/mitochondrial antiviral-signaling protein pathway followed by activation of interferon regulatory transcription factor (IRF)3 or IRF7 in prostate cancer cells (27). HVJ is a mouse parainfluenza virus that belongs to the paramixoviridae genus. Two glycoproteins, HN and F, are present on the viral envelope (28). An infection occurs when HN binds to its receptor followed by the fusion of the hydrophobic region of the F protein to the lipid bilayer through its association with lipid molecules, such as cholesterol (29). After this first infection step, the HVJ virus genome is recognized by RIG-I, and death signaling effectors are activated (30, 31). However, no RIG-I expression was detected in the SK-N-SH cells in this study (data not shown). Therefore, we hypothesized that some mechanism that does not involve the innate immune system is associated with HVJ-E–induced necroptosis. It has been reported that HVJ-E enhances the cytoplasmic Ca^{2+} concentration of target cells upon fusion with the cell membrane, (28, 32–35) and we could confirm this increase in intracellular Ca^{2+}.

Many intracellular Ca^{2+} effects are mediated by protein phosphorylation events that are catalyzed by a family of serine/threonine protein kinases called CaM kinases. Some CaM kinases phosphorylate gene regulatory proteins, such as CREB, and they regulate the transcription of specific genes. One of the best-studied CaM kinases is CaMK II, which is expressed in most animal cells and is especially abundant in the nervous system. It contributes up to 2% of the total protein of some regions in the brain and is highly concentrated in the synapses. CaMK II has a remarkable property in that it can function as a molecular memory device, switching to an activated state when exposed to Ca^{2+}/calmodulin and then remaining activated (36, 37). This is because the kinase phosphorylates itself (autophosphorylation) and it also phosphorylates other proteins following activation by Ca^{2+}/calmodulin. In this autophosphorylated state, the enzyme remains activated even in the absence of Ca^{2+}, thereby prolonging the duration of its kinase activity. The enzyme maintains this activity until serine/threonine protein phosphatases suppress phosphorylated CaMK II and shut it off. CaMK II activation can thereby serve as a memory trace of a prior Ca^{2+} pulse, and it seems to have a role in some types of memory and learning in the vertebrate nervous system. We herein demonstrated that HVJ-E enhances the cytoplasmic Ca^{2+} concentration for a short time followed by the phosphorylation of CaMK II for a longer period. This finding suggests that the temporal increase in cytoplasmic Ca^{2+} can trigger CaMK II autophosphorylation, thereby causing necroptosis via RIP1 phosphorylation in caspase-8–deficient neuroblastoma cells. The first step in RIP1 activation is the deubiquitination of RIP1 by CYLD. RIP1 can then freely migrate to the cytoplasm and form a complex with RIP3. Although little is known about how this complex formation is regulated, it was recently reported that RIP1–RIP3 complex formation requires RIP1 deacetylation by NAD-dependent deacetylase SIRT2 (38). The final step of RIP1 activation requires the phosphorylation of serine 161 to produce ROS through the mitochondrial oxidative phosphorylation pathway, but the factor that phosphorylates RIP1 has not yet been discovered. Although it is not clear whether the regulation of RIP1 by CaMK II is direct or indirect, we are the first to discover one of the downstream Ca^{2+} signaling pathways associated with necroptotic cell death.

In conclusion, an increase in the cytoplasmic Ca^{2+} concentration induces necroptosis via CaMK II phosphorylation in caspase-8–deficient neuroblastoma cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Nomura, M. Fukuzawa, Y. Kaneda
Development of methodology: M. Nomura, Y. Kaneda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Nomura, A. Ueno
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Nomura, Y. Kaneda

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Writing, review, and/or revision of the manuscript: M. Nomura, Y. Kaneda
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Nomura, K. Saga, Y. Kaneda
Study supervision: Y. Kaneda

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Desmoglein as a target in skin disease and beyond

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Abstract

Much of the original research on desmosomes and their biochemical components was through analysis of skin and mucous membranes. The identification of desmogleins 1 and 3, desmosomal adhesion glycoproteins, as targets in pemphigus, a fatal autoimmune blistering disease of the skin and mucous membranes, provided the first link between desmosomes, desmogleins, and human diseases. The clinical and histological similarities of staphylococcal scalded skin syndrome or bullous impetigo and pemphigus foliaceus led us to identify desmoglein 1 as the proteolytic target of staphylococcal exfoliative toxins. Genetic analysis of striate palmoplantar keratoderma and hypotrichosis identified their responsible genes as desmogleins 1 and 4, respectively. More recently these fundamental findings in cutaneous biology were extended beyond the skin. Desmoglein 2, which is expressed earliest among the four isoforms of desmoglein in development and found in all desmosome-bearing epithelial cells, was found to be mutated in arrythmogenic right ventricular cardiomyopathy and has also been identified as a receptor for a subset of adenoviruses that cause respiratory and urinary tract infections. The story of desmoglein research illuminates how dermatologic research originally focused on one skin disease, pemphigus, has contributed to understanding biology and pathophysiology of many seemingly unrelated tissues and diseases.

Keywords

pemphigus; impetigo; hypotrichosis; cardiomyopathy; cadherin

Introduction

The story of the discovery of desmogleins in desmosomes, their relationship to adhesion molecules, and their targeting in diseases with loss of adhesion of cells is one of beautiful simplicity and logic, in which the research in skin has played a major role. This review will tell that story (Table 1).

The story starts with the discovery of desmosomes by electron microscopy and observations that they play a role in adhesion of keratinocytes. Dermatologists already know intuitively that desmosomes are important in cell adhesion, because in spongiosis, one of the most common pathologies seen in inflammatory skin diseases such as allergic contact dermatitis, there is edema between keratinocytes separating their cell membranes, yet the cells stay attached right at the desmosomes (also called in the past "intercellular bridges"). Only when the desmosomes finally "dissolve" does a spongiotic blister form. It was in skin and stratified squamous epithelial tissue in which the presence and importance of desmosomes

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was discovered, but subsequently they were discovered in non-epithelial tissues such as heart (see below).

Biochemical and molecular characterization of desmogleins

Once a method of isolating desmosomes from epithelia (cow's snout was the major source) was established, they could be biochemically characterized. The central part of the desmosome, as seen by electron microscopy as a white area between the cell membranes of adjacent cells, was thought to be the "glue" that held the desmosomes (and, thereby, the cells) together. When this area was enriched from the isolated desmosomes, its protein composition could be determined. A major protein was identified of about 160 kd, and was called desmoglein, "glein" derived from the Greek word for "glue".

Subsequently antibodies against desmoglein were developed. These antibodies indicated that desmoglein was found in desmosomes of various tissues across various species. Desmoglein, therefore, was determined to be a widespread, and presumably important (i.e. conserved through evolution), component of the desmosome. From this work, initially in epidermis, it was found that desmoglein was also found in many other tissues such as intestine, mammary gland, trachea, bladder, liver, and, perhaps unexpectedly, heart and thymus.

Protein isolation and antibodies allowed molecular cloning of desmoglein. Surprisingly, desmoglein was found to be a group of homologous molecules encoded by a gene family. The most interesting, and scientifically satisfying, finding was that desmogleins were in a supergene family defined by classical cadherins, which were already known to be calcium-dependent adhesion proteins. Thus, desmogleins, thought the be part of the "glue" in desomomes, were found to be in a family of adhesion molecules; a very nice convergence of deductions from early morphologic findings and newer genetic cloning techniques.

The first two members of the desmoglein gene family were identified by genetic cloning as desmoglein 1 and 2, but a dermatologic disease, pemphigus vulgaris, allowed identification of a third member (see below), desmoglein 3. Analysis of the human genome database identified another desmoglein, desmoglein 4, which was also identified by mutational analysis of a genetic hair disease (localized autosomal recessive hypothrichosis) (Table 1).

Even at this early stage, characterization of a desmoglein gene family was closely intertwined with understanding skin diseases. However that interdependency reached another level when the relationship of pemphigus to desmogleins was defined.

Desmogleins identified as targets of autoantibodies in pemphigus

There are two major types of pemphigus, vulgaris and foliaceus. In both, blisters are caused from loss of cell adhesion in the deep epidermis or superficial epidermis, respectively. Pemphigus vulgaris affects mucous membranes and/or skin, whereas pemphigus foliaceus only affects skin. In both, autoantibodies are known to directly mediate the loss of cell adhesion.

The original discovery that these diseases are related to desmosomes depended on a precious commodity, *time*, that today (with increasing demands related to extensive grant writing; extensive regulation; the geometric progression of required educational modules for; routine clinical, laboratory and animal work; demands to earn their salaries by seeing patients; and more) is much less available. But the availability of time was the critical resource that allowed one of the authors (JRS), in early 1980, to attend a lecture by Malcolm Steinberg at Dulles Airport, 40 minutes from where I worked in Bethesda. At that time, I had been

working on identifying the autoantigen in pemphigus foliaceus and knew it was an approximately 160 kd protein as identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of epidermal extracts, followed by immunoblotting. This was precisely the same time that Malcolm Steinberg (with G. Gorbsky) was dissecting the proteins in the "desmoglea" (the electron lucent center) of the desmosome. There was no evidence then that pemphigus had anything to do with desmosomes, but since cells come apart in pemphigus I was interested in cell adhesion and, therefore, desmosomes. Since I had enough time to take several hours to attend that talk, I learned that Dr. Steinberg had identified the first desmoglein, now called desmoglein 1, as a 160 kd glycoprotein. Naively, I thought that it would make sense if desmoglein 1 was the pemphigus foliaceus antigen, because both were the same size as determined by SDS-PAGE. (Ignorance is also sometimes a great advantage to a scientist; I had no idea how many molecules extracted from epidermis might migrate in the same general area on a one dimensional SDS-PAGE gel).

In fact, the pemphigus foliaceus antigen was ultimately shown to be desmoglein 1 (Figure 1), and desmoglein 1 was shown to be in the superficial epidermis. This finding showed the first autoimmune disease of desmosomes, and had the scientifically satisfying logic that an antibody against a presumed cell adhesion molecule in a cell adhesion structure causes a disease from loss of cell adhesion and subsequent blister formation.

At the time the pemphigus foliaceus antigen was shown to be desmoglein 1, the pemphigus vulgaris antigen was only known to be a glycoprotein of about 130 kd, as determined by immunoprecipitation. In addition, it was known there was some relationship of the pemphigus vulgaris antigen to desmosomes because it was shown by co-immunoprecipitation that pemphigus vulgaris antigen co-precipitated plakoglobin with the 130 kd molecule. Similarly pemphigus foliaceus sera co-precipitated plakoglobin with desmoglein 1. Plakoglobin was known to be in the plaque of the desmosome inside the cell. These studies were the first to show that the tail of desmogleins (the part inside the cell) bound a plaque protein of the desmosome. Again, a skin disease, pemphigus, was intertwined with our growing understanding of desmosomes, in this case their molecular structure.

What really brought all these observations together, in a beautiful and logical synthesis of previous findings, was the molecular cloning of pemphigus vulgaris antigen which showed it was another, previously unknown, desmoglein, now called desmoglein 3 (Figure 2). Although desmoglein 1 and 3 were both found in epidermis, they were at different levels; desmoglein 1 was superficial and desmoglein 3 was deep. All the previous observations and findings now fit together nicely: Pemphigus vulgaris and foliaceus were closely related diseases that both had loss of keratinocyte adhesion but in different tissue localizations. The autoantibodies bound closely related molecules thought to provide the "glue" in adhesion structures, the desmosomes, with resultant loss of adhesion and blisters. In pemphigus vulgaris and foliaceus the blisters were thought to occur in different tissue localizations because of the different localizations of the desmogleins. Finally, both pemphigus antigens were found to bind plakoglobin because desmogleins bind plakoglobin by their homologous tails.

Many more subsequent studies confirmed that the anti-desmoglein antibodies in pemphigus patients cause the disease. For example, adsorption of pemphigus sera with recombinant desmogleins resulted in loss of pathogenicity of those sera. Monoclonal anti-desmoglein antibodies cause disease when injected into neonatal mice or human skin organ culture (Figure 3). An interesting confirmation that loss of desmoglein 3 adhesion causes pemphigus is that mouse with a genetic deletion of desmoglein 3 develop oral and skin lesions with the

typical histology of pemphigus vulgaris. Finally, specific proteolytic cleavage of desmoglein 1 caused lesions in epidermis histologically indistinguishable from pemphigus foliaceus (see below).

Desmogleins used for the diagnosis of pemphigus

cDNA isolation of desmoglein 1 and 3 allowed us to produce recombinant proteins which properly reflect their three dimensional structures by baculovirus or mammalian expression. The immunoadsorption of patients' sera with those recombinant proteins removed their immunoreactivities on keratinocyte cell surfaces by immunofluorescence and their ability to induce blister formation in neonatal mice. Subsequently, enzyme-linked immunosorbent assay (ELISA) using recombinant desmogleins 1 and 3 were developed as a serological diagnostic tool for pemphigus. Patients with pemphigus foliaceus show only anti-desmoglein 1 IgG autoantibodies, while patients with mucosal dominant type of pemphigus vulgaris have only anti-desmoglein 3 IgG. Finally, patients with the mucocutaneous type of pemphigus vulgaris have both anti-desmogelin 3 and anti-desmoglein 1 IgG. ELISA is a powerful and objective assay that allows easy screening of large numbers of sera to characterize patients for diagnosis and research purposes.

In general, ELISA antibody titers fluctuate in parallel with the disease activity with time in any particular patient, while the titers do not necessarily reflect the disease severity among different patients. This discrepancy is because patients' sera are polyclonal containing both pathogenic and non-pathogenic antibodies for blister formation, as demonstrated by isolation of monoclonal antibodies from mice and humans. Such pathogenicity, or the lack thereof, can be measured in neonatal mice, in skin organ culture or in keratinocyte dissociation assays of cultured human keratinocytes.

Pathophysiology of blister formation in pemphigus

There are two major, not necessarily exclusive, theories of how pemphigus antibodies cause blisters. One theory is that antibodies cause steric hindrance of the desmoglein adhesion site thus interfering directly with adhesion. The other theory is that antibodies cause intracellular signaling that leads to loss of adhesion.

Pemphigus antibodies cause steric hindrance

Epitope mapping of pemphigus autoantibodies and monoclonal antibodies from mice and humans has demonstrated that pathogenic antibodies bind to the amino-terminal extracellular domain of desmogleins that is predicted to form the trans-adhesive interface between cells, based on the crystal structures of classic cadherins, molecules in the same gene family as desmogleins. In addition, pathogenic pemphigus foliaceus monoclonal antibodies cloned from patients bind to the mature desmoglein 1 which reveals the active adhesion site only after the proprotein is cleaved, but not to the proprotein which does not form the adhesion site. So-called "knockout mice" lacking desmoglein 3 show similar, if not identical, acantholytic lesions mimicking the phenotype of mucosal dominant type of pemphigus vulgaris. These mice show that lack of function (i.e. adhesion) of desmoglein 3, without *antibody-induced* signaling (e.g., by crosslinking a cell surface receptor), show the typical histology of pemphigus vulgaris. Desmoglein compensation theory logically explains the site of blister formation in the skin and mucous membranes in patients with pemphigus, suggesting blisters are formed as a result of the loss of adhesive function of each desmoglein isoform. If antibody signaling caused loss of adhesion, then one would expect that whenever antibodies bound desmoglein 3 a blister should result because of the resultant signaling, yet in fact this is not the case wherever desmoglein 1 is also present. These observations suggest that pemphigus blisters are initially caused by steric hindrance and not by the activation of a

signaling pathway that causes the initial loss of adhesion, although such signaling may occur after loss of adhesion and may amplify the initial loss of cell adhesion. Furthermore, these data do not negate that modulation of signaling pathways that control desmosome stability could be useful in counteracting pemphigus antibody-induced loss of adhesion.

Pemphigus antibodies cause intracellular signaling

On the other hand, when pathogenic pemphigus antibodies bind to the keratinocyte cell surface, several signaling events have been shown to take place. The most widely studied are those pathways that involve p38 mitogen-activated protein kinase (p38MAP kinase) and plakoglobin. The most general indication of signaling is that pemphigus antibodies cause protein phosphorylation changes in keratinocytes. Studies of these changes indicated that p38MAP kinase was a major pathway stimulated by both pemphigus vulgairs and pemphigus foliaceus autoantibodies. These pathways may be activated only after cells lose their adhesion, but, even so, they probably increase the acantholytic effects of pemphigus antibodies, and blocking them may improve disease (see below). Another observations regarding signaling in pemphigus was that keratinocytes genetically engineered to lack plakoglobin were not as susceptible to pemphigus antibodies as were wild type cells. This observation led to a pathway involving c-myc, which was shown to be elevated in pemphigus antibody-stimulated keratinocytes.

Some of these signaling pathways are probably involved in normal homeostasis of desmosomes and their components, such as transport to the cell surface, into the desmosome, and internalization. Pemphigus antibodies may act through modulation of these physiologic pathways. For example, as a result of signals induced by pemphigus antibodies that cause loss desmoglein adhesion as desmogleins reach the cell membrane, desmogleins may be depleted first from the cell membrane thereby decreasing or eliminating the pool of desmogleins for their incorporation into desmosomes, ultimating resulting in depletion of desmogleins in desmosomes leading to desmosome dysfunction (i.e. acantholysis). Although it is difficult at this time to detemine which are the most critical signaling pathways contributing to pathogenicity, signaling pathways may be an important target in designing therapy for pemphigus in the future (see below).

Immunology of development of anti-desmoglein antibodies

Why do patients with pemphigus produce harmful autoantibodies against desmogleins? This is an ultimate and fundamental question that we need to answer in the future. To begin to address this question, upstream events of pathogenic antibody production have been investigated. It is probable that both anti-desmoglein B cells and desmoglein-peptide specific T cells that provide help to those B cells are necessary for anti-desmoglein antibody formation. In support of this model, circulating B cells producing anti-desmoglein 3 antibodies were detected by enzyme-linked immunospot (ELISPOT) assay in patients. Circulating T cells reacting with desmoglein 3 were detected in patents, but perhaps surprisingly, were also detected in normal people. To investigate the formation of anti-desmoglein antibodies in an animal model, an active disease mouse model for pemphigus vulgaris was developed by adoptive transfer of lymphocytes from desmoglein 3 deficient mice to immune-deficient desmoglein 3-expressing mice. This model was used to isolate anti-desmoglein 3 pathogenic monoclonal antibodies as well as desmoglein 3-specific T cells that help B cells to produce pathogenic antibodies. Both the T and B cells were needed for antibody formation. Aire (autoimmune regulator, a transcription factor) was also shown to play a role in regulating desmoglein 3 expression in the medullary thymic epithelial cells and in selection of T cells in thymus. Presumably expression of desmoglein 3 in thymus helps provide tolerance to that antigen in most people.

To further clarify the mechanisms for central and peripheral tolerance to desmoglein 3-reacting T cells and B cells will be an important step to develop antigen-specific immunosuppressive therapies.

Desmogleins are targets in infectious diseases

The story of the identification of desmoglein 1 as the target of staphylococcus exfoliative toxin (Table 1) illustrates the importance of having physicians as scientists.

The blisters in bullous impetigo and staphylococcal scalded skin disease have been known for many years to be caused by a toxin made and secreted by staphylococcus aureus, called exfoliative toxin. In bullous impetigo, the toxin is produced locally, causing blisters at sites of infection. In staphylococcal scalded skin syndrome, which usually is seen in infants or very young children, the toxin circulates and causes blisters distant from sites of infection. When injected into mice this toxin causes blisters. However, for many years after is discovery, it was not clear how this toxin caused blisters in the epidermis. Even after the toxin was molecularly cloned and shown to have the structure of a serine protease (i.e. a protease with a serine in its active site) that seemed to need a specific substrate in order to orient its catalytic site to be active, that substrate could not be identified. Ultimately, it was dermatologists, with their knowledge of skin diseases, who were able to solve this longstanding puzzle.

Even as early as the 18th century, astute clinicians who were superb morphologists (probably because they had little technology beyond their eyes and ears) recognized that staphylococcal scalded skin syndrome resembled pemphigus. Because of this observation, staphylococcal scalded skin syndrome was sometimes called "pemphigus neonatorum". In addition, it was known that injection of exfoliative toxin into neonatal mice caused blisters whose histology was identical to that resulting from injection of pemphigus foliaceus antibodies into mice. In fact, any practicing dermatologists knows that whenever a biopsy of a patient with pemphigus foliaceus is taken, the report always indicates that although the histology is consistent with pemphigus foliaceus it is also consistent with bullous impetigo or staphylococcal scalded skin syndrome. Once it was learned that pemphigus foliaceus was caused by antibodies against desmoglein 1, it was a small step in logic to hypothesize that exfoliative toxin's protein substrate might be desmoglein 1. In both cases the function of desmoglein 1 would be impaired with similar resultant histology.

In fact, that simple but eloquent hypothesis was shown to be correct. Exfoliative toxin was shown to cleave one peptide bound in one substrate, desmoglein 1 (Figure 4). The use of this toxin to enhance staphylococcus pathogenicity and infectivity is beautiful in its simplicity. Essentially, staphylococcus has evolved to produce a toxin that targets one peptide bond of one molecule to enable it to grow under the stratum corneum (the barrier of the skin), but enables it to be superficial enough so that when one child touches another the bacteria can be passed on.

Adenovirus provides another example of desmoglein involvement in infection. Most of human adenoviruses (serotypes A to F) use the coxsackie adenovirus receptor as a primary attachment receptor. However, serotype B adenovirus, which cause respiratory and urinary tract infections, does not use this receptor, but has recently been found to bind to desmoglein 2 as the primary high-affinity receptor (Table 1). Adenovirus binding of desmoglein 2 triggers an epithelial-to-mesenchymal-like transition, leading to transient opening of intercellular junctions and penetration of virus into the subepithelial cell layers and the blood stream, allowing the virus to spread.

Desmogleins are targets in genetic diseases

Mutations in genes encoding desmogleins have also been described in skin, hair, as well as heart diseases (Table 1). Striate palmoplantar keratoderma, an autosomal dominant disorder, is characterized by longitudinal hyperkeratotic lesions on the palms associated with focal or diffuse thickening of the plantar skin. Haploinsufficiency mutations in the gene for desmoglein 1, desmocollin 2, or desmoplakin, have been shown to underlie this skin disorder. Mutations in the gene encoding desmoglein 4 cause localized autosomal recessive hypothrichosis in humans and the lanceolate hair phenotype in mice. More recently, it has been discovered that arrhythmogenic right ventricular cardiomyopathy, which is clinically characterized by right ventricular enlargement and dysfunction, fibrofatty replacement of myocytes in the right ventricle, characteristic electrocardiographic abnormalities, ventricular arrhythmia, and sudden death, is caused by mutations in the genes for desmoglein 2, desmocollin 2, plakoglobin, plakophilin 2, or desmoplakin. These genetic findings underscore the importance of desmoglein (and other desmosomal molecules) mediated adhesive function in tissue integrity and function.

Desmogleins do more than provide adhesion of cells

Experiments showing that misexpression of desmoglein isoforms where they are not normally present causes differentiation defects indicate that desmogleins may do more than simply provide adhesion. For example, desmoglein 3 expression in the superficial epidermis, where it is not normally present, cause abnormal epidermal differentiation and statum corneum formation with increased transepidermal water loss. In mice with a genetic deletion of desmoglein 2 there is a defect in blastocyst proliferation resulting in failure of implantation. Conversely, forced expression of desmoglein 2 in the superficial epidermis causes increased proliferation. The above findings indicate profound effects of desmogleins on keratinocyte proliferation and differentiation. Presently, it is thought that these effects are produced through signal transduction pathways, although the exact pathways have not been elucidated. One major contributor to signaling through desmosomes may be plakoglobin. As discussed above, desmogleins bind to plakoglobin, which is related to β-catenin, a well characterized molecule important in the Wnt signaling pathway. This pathway is important in proliferation and differentiation as well as embryological development. Amount and isoforms of desmoglein may control amount of free plakoglobin that could contribute to signaling. However, much work needs to be done in the future to link signaling to specific molecules and pathways.

Further evidence indicating that desmogleins do more than just provide cell adhesion are the observations that demonstrate that desmoglein 1 is critical for proper stratification and differentiation of epidermis. Interestingly, this effect, which is associated with down regulations of the EGFR-Erk1/2 signaling pathway, requires neither extracellular adhesion nor intracellular plakoglobin binding. These results implicate pathways independent of plakoglobin in desmoglein signaling.

The future: therapy of pemphigus based on the understanding of its pathophysiology and immunology

Anti-idiotypic therapy

Recent genetic analysis of monoclonal antibodies cloned from pemphigus vulgaris and foliaceus patients indicates that both pathogenic and non-pathogenic monoclonal antibodies are found, and that there are a limited number of parenteral B cell clones for these antibodies. A limited set, but several different, immunoglobulin variable heavy chain genes are used in patients to make pathogenic antibodies, therefore, it probably is not possible to

target variable heavy chains to treat disease. However, when the complementarity determining region 3 (CDR3) of the antibodies was analyzed, pathogenic antibodies shared a common sequences. (The CDR3 region of antibodies is thought to be the major region that binds the corresponding antigen). Randomization and site-directed mutagenesis of the heavy chain CDR3 sequences in some of these antibodies illustrated the importance of only a few, or even one, amino acids for binding and/or pathogenicity. Such data suggest that it might be possible to target common sequences in the CDR3 regions of pathogenic pemphigus antibodies to prevent disease. Reagents derived from phage display cloning of monoclonal antibodies from patients could be used to screen for such blockers.

Rituximab

From knowledge that pemphigus is an autoantibody-mediated disease with a limited set of anti-desmoglein non-tolerant B cells, as discussed above, it makes sense that eliminating B cells might improve disease, especially if these non-tolerant set of B cells cannot come back after B cell depletion. In fact, the success of rituximab for therapy tends to validate this idea. Rituximab is an anti-CD20 antibody. CD20 is found on all mature B cells, but not the stem B cells. B cell pools are re-populated from stem B cells (i.e. B cells that have not formed immunoglobulins against specific antigens). Rituximab should, therefore, in theory, eliminate anti-desmoglein B cell clones.

In fact, rituximab has shown excellent results in treating pemphigus patients refractory to standard therapy (e.g. prednisone plus immunosuppressives like azathioprine or mycophenolate mofetil). A single cycle (2 injections over 2 weeks or 4 injections over a month) can lead to complete remission is over 80% of patients, although some relapse and some may need low dose prednisone therapy. Rituxan causes a profound depletion of circulating B cells, but presumably does not deplete plasma cells, which do not have CD20 on their surface. Since in most patients anti-desmoglein antibodies go down after rituximab therapy, the anti-desmoglein antibody-producing plasma cells may be short-lived and need frequent replenishment by B cells.

In any case, elimination of circulating B cells has proven to be excellent therapy for patients with refractory pemphigus.

Signaling

As discussed above, pemphigus antibody binding to desmoglein on the keratinocyte cell surface causes signal transduction which can exacerbate acantholysis. Therefore, if the right signaling pathways can be identified then pharmacologically interrupting them might be beneficial in patients. For instance, blocking the p38MAP kinase pathway in a mouse model of pemphigus has been shown to modulate disease.

In addition, studies, discussed above, have shown that the normal system of cycling of desmosomal components may be interrupted in pemphigus, causing depletion of desmoglein in desmosomes with subsequent acantholysis. Tools might be found to perturb signaling to affect this physiologic process in order to increase desmoglein synthesis and subsequent incorporation into desmosomes, thereby, stabilizing them and counteracting the depletion induced by pemphigus antibodies. It may be that corticosteroids, which are already known to be very effective in therapy, act by this precise mechanism, in that they increase desmoglein synthesis through signaling.

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