Acute Skin Eruptions That Are Positive for Herpes Simplex Virus DNA Polymerase in Patients With Stem Cell Transplantation

A New Manifestation Within the Erythema Multiforme Reactive Dermatoses

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Background: Patients with stem cell transplantation (SCT) develop erythematous eruptions (SCTE) that are often misdiagnosed and poorly treated. Latent herpes simplex virus (HSV) is likely to be reactivated by SCT-associated immunosuppression. Therefore, one of the differential diagnostic possibilities for SCTE is HSV-associated erythema multiforme (HAEM) in which HSV genetic fragments localize in stem cells that deliver them to the skin on differentiation.

Observations: Lesional skin from patients with SCTE, HAEM, HSV, or drug-induced erythema (DIEM) was stained with antibodies to the HSV antigen DNA polymerase (Pol) and the major capsid protein, virion protein 5 (VP5). The HSV DNA polymerase Pol was expressed in 79% of patients with SCTE and 75% of those with HAEM. The protein VP5 was not expressed in these patients, indicative of the absence of virus replication. Findings in patients with DIEM were negative for both antigens, and those with HSV lesions were positive for both antigens.

Conclusions: There is a growing problem with SCTE, related to the increasing numbers of performed SCT. The greater frequency of SCT-generated circulating stem cells in patients with hematological malignant neoplasms (who have latent HSV infection) may result in a widespread SCTE characterized by skin deposition of HSV DNA fragments, notably those expressing Pol antigen. This HAEM-like presentation should be considered in the differential diagnosis of SCTE. Prolonged high-dosage antiviral chemotherapy during and after hospitalization may be warranted.

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HERPES SIMPLEX VIRUS (HSV)-associated erythema multiforme (HAEM) falls into a clinical spectrum that ranges from mild erythema multiforme (EM) through the severe disorders of Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).1-3 The molecular hallmark of HAEM is the presence and expression of the HSV DNA polymerase gene (Pol) in lesional skin4-14 and circulating CD34+ stem cells.15 The CD34+ cells are increased in number in peripheral blood collected during acute HAEM episodes,15 suggesting that endogenous stem cells contribute to HAEM pathogenesis. Because the numbers of circulating CD34+ cells are increased in patients who receive stem cell transplants (SCTs) for hematological malignant neoplasms, we wanted to know whether these patients are at increased risk of developing erythematous lesions consistent with HAEM. A large proportion of these transplant patients had HAEM-like eruptions, which we defined as SCT erythema (SCTE), that were positive for the Pol antigen.

METHODS

SUBJECTS

Twenty adult patients were observed from 2003 to 2006. They were selected sequentially from the Dermatology Service records of post-SCT consultations for skin eruptions requiring biopsy. All individuals had received an allogeneic or autologous SCT for a hematological cancer. The cancer treatment protocol was terminated the day before transplantation. On the day of hospitalization, oral acyclovir, 800 mg, twice daily, and immunosuppressive treatment were initiated. The patients developed SCTE within 3 to 15 days after transplantation. Punch biopsies were performed on active lesions from these patients. Biopsy samples were also taken from eroded epithelium in the esophagus and stomach from 1 patient and peripheral blood mononuclear cells (PBMCs) were obtained from 50 mL of blood collected from another patient at the time the SCTE lesions exacerbated. Biopsy samples of normal
perilesional skin were obtained from 2 patients with SCTE. Specimens of cutaneous lesions were also obtained from 24 adult patients with a clinical diagnosis of HAEM following an established HSV recurrent episode confirmed by histopathologic diagnosis of the skin biopsy and positive findings from immunohistochemical analysis for interferon γ (IFN-γ), as previously described. Lesional skin samples were also obtained from 5 patients with clinically diagnosed HSV lesions confirmed by virus isolation and from 20 patients with a bullous reactive eruption secondary to drug intake and unrelated to HSV infection, which we designated as drug-induced erythema multiforme (DIEM). Normal skin was also obtained from 4 healthy controls. The medical ethics committee of the University of Maryland, Baltimore, approved all the described studies, which were conducted according to Declaration of Helsinki principles. Participants gave their written informed consent.

ANTIBODIES

The Pol antibody was generated in rabbits using an HSV-type common peptide located at residues 1216 to 1224 of the HSV-1 Pol protein and was used as previously described. The phycoerythrin-conjugated mouse monoclonal antibody to human CD34 antigen was obtained from BD Biosciences Pharmingen (San Diego, California). Secondary antibodies were Alexa-Fluor 546– and Alexa-Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon), Texas Red–labeled horse anti-mouse IgG (Vector Laboratories, Burlingame, California), and horseradish peroxidase–conjugated goat anti-mouse IgG (Cell Signaling Technology, Beverly, Massachusetts). The respective specificities of all antibodies are established. Normal rabbit IgG was prepared in the laboratory and used as a negative control. Antibody to the major HSV capsid protein VP5 was obtained from Virusys Corp (Sykesville, Maryland) and used to detect virion formation as a result of virus replication.

IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMICAL ANALYSIS

Paraffin-embedded tissues were sectioned at 5 µm. Slides were deparaffinized in xylene and ethanol gradients, washed in phosphate-buffered saline (PBS), and subjected to antigen retrieval using RetrieveA (pH 6.0: BD Bioscience, San Jose, California) in a 90°C water bath for 10 minutes. Slides were allowed to cool in RetrieveA solution (20 minutes at 25°C), washed with PBS, and blocked (1 hour at 25°C) in blocking buffer (5% normal goat serum and 5% body surface area in PBS). Slides were incubated with primary antibodies (18 hours at 4°C) in blocking buffer and washed with 0.1% Tween-20 in PBS followed by biotinylated secondary antibody (1 hour at 37°C). For immunohistochemical analysis, they were subsequently exposed to avidin-conjugated alkaline phosphatase and alkaline phosphatase substrate (LSAB-2 AP kit; Dako Corp, Carpenteria, California) according to the manufacturer’s instructions. For immunofluorescence, the secondary antibodies were conjugated to Texas Red, Alexa-Fluor 488, or Alexa-Fluor 546. The tissue sections were subsequently washed, mounted in Vectashield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories), and visualized with a fluorescent microscope (model E4100; Nikon USA, EP, Segundo, California) utilizing fluorescence isothiocyanate (FITC) (330-380nM) and UV (for DAPI) (465-495nM) cubes.

COLLECTION OF CD34+ CELLS AND FLOW CYTOMETRY

We isolated the PBMCs using discontinuous Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. They were stained with CD34 antibody and analyzed by flow cytometry, as previously described. In addition, PBMCs (20 µL) were smeared on glass slides, allowed to air dry, and stained with Alexa-Fluor 546–labeled CD34 and Alexa-Fluor 488–labeled Pol antibodies in double immunofluorescence.

RESULTS

The patients who developed erythematous lesions pursuant to SCT had several common features. They received an allogenic or autogenic SCT for serious hematological diseases, including acute myelogenous leukemia, chronic myelogenous leukemia, and multiple myeloma. All medications were discontinued the day prior to transplantation. Oral acyclovir was administered together with the immunosuppressant on the day of hospitalization and continued for several weeks. Within 3 to 15 days after the procedure, 19 of 20 studied patients developed a generalized, bilaterally symmetrical, erythematous, maculopapular eruption, which had a predilection for acral regions. Its severity increased within a few additional days, resulting in the formation of plaques, which darkened and later desquamated. Some of the lesions had a targetoid configuration, or included small peripheral microvesicles or pustules. Rarely, there was a suggestion of urticaaria at the edges of the involved regions. There were no bullae, grouped vesicles, or ulcers. Early lesions were pruritic and later could be painful or tender. The dermatological consultations on these patients occurred on days 3 to 15 of the in-hospitalization stay (Figure 1).

Pol-POSITIVE LESIONAL SKIN FROM PATIENTS WITH SCTE

A positive control for our studies of SCTE is 1 of the 20 studied patients with SCT who, despite acyclovir therapy, developed a generalized vesicular rash that was accompanied by neck stiffness and vomiting and was diagnosed as having generalized systemic HSV infection. An
intraepidermal vesicle with numerous eosinophilic rounded viral inclusion bodies and multinucleated giant cells was seen on a biopsy specimen from the pretibial area of this patient. The skin lesion (encompassing the vesicle site) stained with antibodies to VP5 (Figure 2A) and Pol (Figure 2B) but not with normal IgG (Figure 2B). Staining was in the epidermis and at the dermal surface of the vesicle’s base. It localized both in the cytoplasm and the nucleus for VP5 and was primarily nuclear for Pol (Figure 2A and B). Similar staining patterns were seen for these antibodies in HSV-2–infected (24 hours postinfection), but not mock-infected, Vero cells (Figure 2C), confirming antibody specificity both in cultured cells and patient tissues.

Biopsy samples of the skin lesions from the other 19 patients who were classified as having SCTE subsequent to transplantation were similarly examined for expression of Pol and VP5 antigens by immunofluorescent staining. Staining with Pol antibody was seen in 15 of 19 patients (79%), as shown for some of these in Figure 3A. Duplicate sections did not stain with VP5 antibody (Figure 3B), indicative of the absence of virus replication, and normal IgG did not stain (Figure 3A). Similar staining patterns were seen in skin lesions from 18 of 24 (75%) patients with HAEM obtained within 10 days of the eruption onset (80% were collected within the first 4 days) (Figure 3A and B). This is in contrast to lesional biopsy samples obtained from 5 patients diagnosed as having HSV by virus isolation, which stained with VP5 antibody (Figure 2) and is consistent with our previous reports for patients with HAEM and HSV.7-11,16 The Pol staining in the HSV and HAEM lesions was intranuclear, as previously reported,7-11,16 but its localization in the SCTE lesions was primarily cytoplasmic (Figure 3A). Perilesional skin samples from patients with SCTE (1-2 cm), normal skin from 4 healthy controls, and skin lesions from 20 patients with DIEM, biopsied on days 3 to 8 of disease onset, did not stain (Figure 3A).

The incidence of Pol antigen expression in lesional skin was unrelated to the patient’s age, sex, time elapsed from diagnosis, site of biopsy, whether the transplant was allogeneic or autologous, the chemotherapeutic protocol, and the duration of acyclovir treatment. In all the Pol-positive patients, staining was localized in the epidermis. Seven patients, whose lesions were particularly severe, also had Pol staining in the dermis (Figure 3A). In these patients, the eruptions involved more than 20% of the body surface, were advancing at the time of biopsy, and were thickened in places (papules and nodules). They were critically ill patients, and their survival was shorter. In 2 patients, biopsy samples were obtained from 1 severe lesion and 1 relatively mild lesion, and only the severe lesion evidenced Pol staining in the dermis, suggesting that Pol expression in the dermis is associated with increased lesion severity. An additional finding was that Pol was absent from mucosal remnants and submucosa.

Figure 2. The DNA polymerase (Pol) and virion protein 5 (VP5) antigens are expressed in the skin lesion from a patient with generalized herpes simplex virus (HSV) following stem cell transplantation. A, Immunohistochemical analysis with VP5 antibody of HSV lesional skin encompasses a microvesicle (left box) that is magnified in the right box (magnification ×400). Staining is cytoplasmic and nuclear and is located in the midepidermis and lower epidermis (arrows). B, Immunofluorescent staining of lesional skin with Alexa-Fluor 546–conjugated Pol antibody or normal IgG. Staining is cytoplasmic (arrow) and in this section is primarily localized in the midepidermis. The 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California) is blue staining. C, Vero cells infected with HSV-2 (18 hours) (left panels) or mock-infected with phosphate-buffered saline (right panels) were stained with Pol antibody and Alexa-Fluor 546–conjugated secondary antibody (Molecular Probes, Eugene, Oregon) (2 top panels, magnification ×600) or VP5 antibody and Texas Red–conjugated secondary antibody (Vector Laboratories) (2 bottom panels, magnification ×600). The DAPI is blue staining.
of the GVH-eroded stomach and esophagus from 1 of the patients with SCTE, indicating that SCTE and GVH can coexist yet be differentiated on the basis of Pol antigen expression.

**EXPRESSION OF Pol IN CIRCULATING CD34+ CELLS**

In patients with HAEM, Pol is expressed in a proportion of CD34+ cells that transport the DNA to distant skin sites. To examine whether Pol can be expressed in CD34+ cells from patients with SCTE, PBMCs collected from 1 patient at the time that the skin lesion was Pol positive were stained by double immunofluorescence with FITC-labeled Pol antibody and Alexa-Fluor 546-labeled antibody to CD34. Approximately 1% of the total CD34+ cells stained with Pol antibody (Figure 4). In addition, fluorescence-activated cell sorter analysis indicated that the mean (SD) percentage of circulating CD34+ cells was elevated (16.8%) relative to previously established values for healthy subjects (2.0% [0.1%]) and those with HSV (3.9% [1.8%]) but similar to the percentages in those with HAEM.

**COMMENT**

Erythema multiforme is a polymorphic, often recurring disease caused by exposure to medication or various infections, notably HSV. Three categories of severe bullous disorders, classified as bullous EM, SJS, or TEN, were suggested as different entities along a spectrum of clinical severity, with SJS and TEN further subdivided by extent of body surface involved and whether large sheets of epidermis were detached. Drug intake was predominant in the more severe end of the spectrum (TEN and SJS), whereas HSV infection seemed to be an important, albeit not the sole, pathogenic factor in the less severe end of the spectrum (EM). The syndrome HAEM is characterized by bilateral erythematous eruptions, the histological features of which include epidermal apoptosis, basal cell degeneration, exocytic cellular accumulation in the superficial epidermis, and mononuclear dermal infiltration. The studies reported by our group over the past 20 years5-13 and confirmed by independent investigations5,13,14 have elucidated the molecular pathogenesis of HAEM, allowing its definition using laboratory markers.

In addition, HAEM is a viral disease with inflammatory and autoimmune components. The lesions in HAEM are virus free but contain HSV DNA fragments, most often comprising sequences that encode and express Pol.5-14 These DNA fragments are generated within circulating CD34+ stem cells that gather virus or viral DNA from the site of a preceding HSV lesion and commonly retain the Pol DNA sequence. The frequency of these CD34+ cells is increased in patients with acute HAEM, and they differentiate with the help of virus upregulated E-cadherin into Langerhans cells as they leave the circulation to enter the skin at peripherial sites where the viral DNA is deposited.13 Viral protein expression in the skin (notably Pol) initiates lesion development through recruitment of a Vβ-restricted population of virus-specific CD4+ helper T cells, type 1, that produce IFN-γ.7,11 This early virus-specific response is followed by an amplified inflammatory cascade, characterized by enhanced cytokine production and the accumulation of T cells that respond to auto-antigens, which are likely released by lysed or apoptotic virus-infected cells. The Pol-induced upregulation of the transcription factor SP1 and SP1-regulated genes in HAEM lesional skin are associated with the inflammatory and autoreactive components of HAEM.

This is a descriptive study of SCTE, the thrust of which is that SCTE is a form of HAEM that often follows SCT. Clinically, most of the patients with SCTE fell into the EM part of the EM/SJS/TEN spectrum,3 although several exhibited generalized lesions, some of which were confluent. The expression of Pol in a high percentage of the SCTE lesions is consistent with this conclusion. Indeed, Pol antigen was seen in lesional skin from 15 of 19 (79%) of those patients with SCTE studied, a frequency similar to that seen in those with HAEM.
sional skin (18 of 24 [75%]), and both were free of infectious virus. These findings, together with the observation that at least 1 patient with SCTE had an increased percentage of circulating CD34<sup>+</sup> cells, some of which were also Pol positive, suggest that SCTE behaves like HAEM. However, numerous questions remain. Is the Pol-positive erythematous eruption in patients with SCT caused by the reactivation of a latent HSV infection when acyclovir was discontinued or administered at a low dosage at or after the time of transplantation? Was the SCT an opportunity for more viral DNA fragments to be disseminated peripherally in the circulation? Definitive answers to these questions are not possible at present. Herpes simplex virus reactivation and its processing by the transplanted CD34<sup>+</sup> cells is likely because up to 80% of patients undergoing a cancer treatment procedure will have HSV reactivation<sup>18</sup> and the percentage of circulating CD34<sup>+</sup> cells, which can transport HSV DNA fragments to the skin, is markedly increased through the SCT procedure. However, 2 caveats remain. The first is the absence of a detectable, localized HSV-like lesion in patients with SCTE. It is possible that the HSV lesions were hidden (for example, in the nasopharynx) or that they were mild or unnoticed because the patients were intermittently treated with antiviral drugs. The chemotherapeutic agents used for the patients’ malignant neoplasms and/or the immunosuppressive drugs prescribed during their illnesses might have dampened the development of clinically visible lesions while allowing for asymptomatic virus shedding, a rather common presentation associated with low virus titers.<sup>7,10</sup> The second caveat to the conclusion that SCTE is a form of HAEM is that Pol staining is cytoplasmic, as opposed to HAEM, where it is also intranuclear. The interpretation of this distinct intracellular localization is still unclear. However, staining is specific because (1) skin tissues from healthy subjects and those with DIEM did not stain, (2) staining was not seen in normal perilesional skin, (3) Pol staining was seen in HSV lesional skin from the patient with SCT with generalized HSV that stained with antibody to VP5 and was positive for virus isolation, and (4) normal IgG did not stain. Cytoplasmic localization may reflect deletion of the intranuclear localization signal during DNA fragmentation and/or the

Figure 4. Lesional skin and circulating CD34<sup>+</sup> cells stained with DNA polymerase (Pol) antibody in a patient with stem cell transplantation with erythematous eruptions (SCTE). A, Lesional skin from a patient with SCTE stained with Pol antibody and Alexa-Fluor 546–conjugated secondary antibody (Molecular Probes, Eugene, Oregon). The Pol staining is diffusely distributed throughout the epidermis and is primarily cytoplasmic (original magnification ×100). B, Lesional skin from the same patient with SCTE stained with VP5 antibody and Texas Red–conjugated secondary antibody (Vector Laboratories, Burlingame, California) (magnification ×100). C, Double immunofluorescent staining of peripheral blood mononuclear cells from this patient with phycoerythrin-conjugated CD34<sup>+</sup> antibody and Alexa-Fluor 488–labeled Pol antibody identifies a small subset of CD34<sup>+</sup>/Pol<sup>+</sup> cells. The 4,6-diamidino-2-phenylindole staining (DAPI; Vector Laboratories) is blue (original magnification ×200 [inset, magnification ×600]).
loss of a nuclear protein that influences Pol’s intranuclear accumulation. Patients with SCTE may also represent a distinct EM subgroup and/or distinct Pol-mediated inflammatory responses. It is our belief that SCTE is not restricted to patients with SCT for hematologic malignant neoplasms and is likely to also apply to other immunosuppressed transplant patients.

Studies of larger patient cohorts and further analysis of their HSV history and immune responses are needed to address the role of HSV reactivation in SCTE and verify the contribution of circulating CD34+ cells. However, bearing in mind that (1) those with HAEM have a delayed clearance of Pol from their body and (2) the oral antiviral therapy used was acyclovir, which has limited oral bioavailability, we assume that the antiviral therapy regimen might have been suboptimal. Whether the antiviral drug should be administered orally or parenterally is debated. At our institution, acyclovir is prescribed only orally because of cost. According to our interpretation, continuous antiviral therapy initiated before admission in higher doses and with improved oral bioavailability should block SCTE development by inhibiting replication of reactivated virus, thereby reducing the number of future DNA fragments. Therapy for many weeks would be necessary to permit cutaneous clearance. The aim of future studies should be to treat with the appropriate antiviral drug so as to eliminate Pol from the skin while assessing the incidence, severity, and morphologic characteristics of eruptions appearing in patients with SCT.

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