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Neutrophils Are a Key Component of the Antitumor Efficacy of Topical Chemotherapy with Ingenol-3-Angelate

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Harnessing neutrophils for the eradication of cancer cells remains an attractive but still controversial notion. In this study, we provide evidence that neutrophils are required to prevent relapse of skin tumors following topical treatment with a new anticancer agent, ingenol-3-angelate (PEP005). Topical PEP005 treatment induces primary necrosis of tumor cells, potently activates protein kinase C, and was associated with an acute T cell-independent inflammatory response characterized by a pronounced neutrophil infiltrate. In Foxn1nu mice depleted of neutrophils and in CD18-deficient mice (in which neutrophil extravasation is severely impaired) PEP005 treatment was associated with a >70% increase in tumor relapse rates. NK cell or monocyte/macrophage deficiency had no effect on relapse rates. Both in vitro and in mice, PEP005 induced MIP-2/IL-8, TNF-α, and IL-1β, all mediators of neutrophil recruitment and activation. In vitro, PEP005 activated human endothelial cells resulting in neutrophil adhesion and also induced human neutrophils to generate tumoricidal-reactive oxygen intermediates. Treatment of tumors with PEP005 significantly elevated the level of anticancer Abs, which were able to promote neutrophil-mediated Ab-dependent cellular cytotoxicity (ADCC) in vitro. PEP005 treatment of tumors grown in SCID mice was also associated with >70% increase in tumor relapse rates. Taken together, these data suggest a central role for neutrophil-mediated ADCC in preventing relapse. PEP005-mediated cure of tumors therefore appears to involve initial chemoablation followed by a neutrophil-dependent ADCC-mediated eradication of residual disease, illustrating that neutrophils can be induced to mediate important anticancer activity with specific chemotherapeutic agents. The Journal of Immunology, 2006, 177: 8123–8132.

The compound ingenol-3-angelate (PEP005) is currently in clinical trials for skin cancer and solar keratoses. PEP005 is a diterpenic ester extracted from the plant Euphorbia peplus, the crude sap of which has a long history of use for various ailments including self-treatment of skin cancers (11). We have shown previously that daily topical applications of PEP005 for 3 days to a number of mouse and human tumors grown s.c. in both C57BL/6 and Foxn1nu mice resulted in tumor cure without significant relapse (12), indicating that T cells are not required for effective anticancer activity. A notable feature of topical PEP005 treatment was induction of an acute erythema, which was associated with a significant infiltration of inflammatory cells, predominantly neutrophils. At high concentrations, PEP005 is acutely cytotoxic to tumor cells, causing cell death by induction of primary necrosis (12). At lower concentrations, PEP005 activates protein kinase C (PKC) (13) and PKC activators are known to stimulate a number of immune responses including induction of proinflammatory cytokines and neutrophil activation (14–16). In this study, we provide evidence that neutrophils are required to prevent relapse following topical treatment with PEP005. Relapse rates were high in two animal models where neutrophil activity was deficient, and both in vitro and in vivo PEP005 was shown to induce mediators associated with neutrophil recruitment and activation. The high relapse rates in SCID mice (B cell deficient), the induction of anticancer Abs by PEP005 treatment, and the ability of such Abs to mediate ADCC with neutrophils in vitro, support the view that neutrophil-mediated ADCC is involved in preventing relapse. Thus, PEP005 appears to have a two stage mechanism of action, initial chemoablation followed by a neutrophil-mediated ADCC-dependent eradication of residual disease.

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Materials and Methods

Cells and cell culture

The B16 mouse melanoma line (CRL-6322; American Type Culture Collection (ATCC)), the LK2 UV-induced mouse squamous cell carcinoma line (17), and the human melanoma lines MM96L (18) and Mel10538 (19) were cultured at 37°C and 5% CO2 in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (CSL Biosciences), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Invitrogen Life Technologies) (complete medium). Human epidermal keratinocytes were isolated from newborn foreskin and cultured in the presence of a mitomycin C-treated 3T3 feeder layer as described previously (20). Human skin fibroblasts were isolated from tissue digests taken at the time of joint replacement surgery and were grown out over several weeks in defined medium as described previously (21). Human neutrophils were isolated from peripheral blood by Percoll density centrifugation as described previously (22) and purity was assessed by Giemsa staining and was routinely >95%.

PEP005 treatment and histology

LK2 cells (10⁶) were injected s.c. (two tumor sites per mouse and two mice per group) into the flanks of 6- to 10-week-old Foxn1nu (BALB/c) mice (Animal Resource Centre, Perth, Australia). The tumors and two patches of normal skin on the opposite flanks were treated once topically with PEP005 or placebo (isopropanol-based gel). PEP005 was dissolved in 100% ace- nyl wax embedded, and processed for histology and standard H&E stain- post-PEP005 treatment and treated sites were excised, formalin fixed, par- assessed by Giemsa staining and was routinely

PEP005 treatment in neutrophil-depleted mice

LK2 cells (10⁶) were injected s.c. (four tumor sites per mouse) into the flanks of 6- to 10-week-old Foxn1nu mice (n = 3 mice and n = 12 tumors/group). After 2 wk (when the tumors had reached ~14 mm³), six mice were given i.p. injections of rat anti-Ly-6G anti-granulocyte Ab (100 μg in PBS) (RB6-8C5; BD Biosciences). Another six mice were injected i.p. with an isotype-matched control Ab (100 μg of rat IgG2b in PBS) (A95-1; BD Biosciences). Ab was injected on days −2, 0, and 2, relative to initiation of PEP005 treatment and treated sites were excised, formalin fixed, par- amines Antigen-1 (RSap1) and C-C motif chemokine ligand 2 (CCL2) expression in PEP005-treated mice. The RNA was purified from skin and tumor tissue as per the manufacturer’s instructions (RNeasy Protect-midi kit; Qiagen). Total RNA (4 g) was reverse tran- scribed using (Oligo d(T)₃) and Superscript III (Invitrogen Life Technolo- gies). PCR product intensity after gel electrophoresis and staining was determined to be linear for the number of cycles used, thus the analysis was deemed to be semiquantitative. The PCR was conducted using the Gene- Amp PCR System 9700 (PerkinElmer), DyNaZyme II DNA polymerase (Finnzymes), and Hot Star TaqDNA polymerase (Qiagen) as described previously (24). The primers were IL-1 RNase Protection assay using total RNA from a parallel group of mice and was adjusted for the mouse effect when four tumors per mouse were used. The analysis showed that the influence of mouse on time to relapse was not significant (p = 0.88 for Fig. 2C and p = 0.315 for Fig. 4D), illustrating that each tumor essentially behaves independently.

In vivo cytokine responses

LK2 cells were injected, as above, into the flanks of 6- to 10-week-old Foxn1nu mice (two tumor sites per mouse). After 2 wk (when the tumors had reached ~14 mm³), the tumors and two skin sites on the opposite flanks of the animals were treated once with PEP005 as above. Parallel mice (n = (time point) were euthanized 0, 1, 2, and 6 h after treatment, and the tumor and skin sites were excised. Total RNA was extracted and purified from skin and treated tissue as per the manufacturer’s instructions (RNasey Protect-midi kit; Qiagen). Total RNA (4 μg) was reverse tran- scribed using (Oligo d(T)₃) and Superscript III (Invitrogen Life Technolo- gies). PCR product intensity after gel electrophoresis and staining was
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Cytokine production by PEP005-treated human cells

Cells of Mel10538 cells, keratinocytes, fibroblasts (75% confluence at ~5 × 10⁵ cells/well of a 24-well plate) and neutrophils (10⁵ cells/well of a 96-well plate) were incubated for 6 h in the absence or presence of PEP005 (1–100 ng/ml). The supernatants were harvested and analyzed for the presence of the following cytokines: TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and GM-CSF using a multiplex detection kit (Bio- Source International).

PEP005-induced activation of vascular endothelium

Adherence of neutrophils to vascular endothelium was measured using a standard static adhesion assay (25). First passage HUVECs were grown to confluent on glass coverslips in the wells of 24-well plates using Medium 199 (Invitrogen Life Technologies) supplemented with 20% FBS (Sigma- Aldrich), 1 mg/ml epidermal growth factor (Sigma-Aldrich), 1 μg/ml hy- drocortisone (Sigma-Aldrich), 28 μg/ml gentamicin (David Bull Labora- tories), and 2.5 μg/ml amphotericin B (Sigma-Aldrich). PEP005 was added to culture wells at 1–100 ng/ml and TNF-α was used as a positive control at 100 U/ml. Endothelial cells were exposed to TNF-α or PEP005 for 4 h before thorough washing to remove PEP005 or TNF-α. Human neutrophils (7.5 × 10⁵/well) were then added to the endothelial cells and allowed to adhere for 5 min at 37°C. Cells were then washed, fixed in 2% glutaral- dehyde, and examined by phase contrast microscopy to determine the num- ber of neutrophils adhering to the endothelial cell monolayer.

MM96L cell killing by neutrophils in vitro

Human neutrophils were added to MM96L human melanoma cells (5000 cells/200 μl of a 24-well plate in triplicate), with and without 10 ng/ml PEP005. After 24 h, the cultures were washed with PBS to remove neutrophils and PEP005 and were then maintained for a further 6 days in complete med- ium. The cells were washed with PBS, fixed in methanol, and the total

B6.129S7-Igh2b(+/−) mice (The Jackson Laboratory) are CD18 hypo- morphic mice on the B6 background and have 2–16% of normal CD18 expression on granulocytes (23). B6 mice (10⁵) were injected s.c. (one tumor site per mouse) into the flanks of 6- to 10-week-old mice (n = 9 mice/group) and after 6 days (when tumors had reached ~14 mm³) mice were treated with PEP005 or placebo. Tumor and erythema size were measured as above.

PEP005 treatment in NK-depleted mice

LK2 cells (10⁶) were injected s.c. (four tumor sites per mouse) into the flanks of 6- to 10-week-old Foxn1nu mice (n = 3 mice and n = 12 tumors/ group). After 2 wk when the tumors had reached ~14 mm³, mice were treated with PEP005 or placebo (on days 0, 1, and 2) and tumor volumes were monitored as above. The mice received no Ab. The tumors on a total of nine mice (n = 3, no Ab; n = 3, A95-1 treated; and n = 3, RB6-8C5 treated) were treated topically daily for 3 days with PEP005 as above. Another nine mice, grouped as above, were treated with the same volume of placebo (isopropyl-based gel). Tumor and erythema size were measured with calipers. Blood was taken from tail tips 5 days after thorough washing to remove PEP005 or TNF-α. Human neutrophils (7.5 × 10⁵/well) were then added to the endothelial cells and allowed to adhere for 5 min at 37°C. Cells were then washed, fixed in 2% glutaral- dehyde, and examined by phase contrast microscopy to determine the num- ber of neutrophils adhering to the endothelial cell monolayer.

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protein of the adherent MM96L cells was determined using sulforhodamine B as described previously (26). Cell survival was expressed as a percentage of total protein measured from wells containing only melanoma cells.

**Lytic mediator release by neutrophils in vitro**

Neutrophils (10^6) were treated with 10 ng/ml PEP005 for 2 h and assessed for generation of 1) superoxides measured using a lucigenin-based assay (27), 2) release of soluble TRAIL measured by ELISA using a commercial kit (BioSource International), and 3) release of defensins 1–3, measured by ELISA using a commercial kit (Cell Sciences).

**Anti-cancer Ab measurements**

B16 cells (10^6) were injected s.c. (one tumor per mouse) into the flanks of 6- to 10-wk-old C57BL/6 mice (Animal Resource Centre) and when tumors had reached ~14 mm^3 they were treated with PEP005 as above. On days 11 and 135, sera was taken and analyzed by ELISA for Abs specific for B16. A group of B16-bearing animals, which were not treated with PEP005 and a naive group were included. B16 cells were sonicated in carbonate buffer (pH 9) and absorbed onto Immuno Maxisorp 96-well plates (Nunc) overnight and dried. The plates were blocked with 5% FBS, 0.01% Tween 20 in PBS for 1 h at 37°C. Test sera was serially diluted in duplicate and probed with rat anti-mouse biotinylated primary Ab (BD Biosciences/BD Pharmingen) and HRP-labeled streptavidin (BioSource International) followed by ABTS substrate (Sigma-Aldrich) and measurement of OD at 405 nm.

**In vitro neutrophil ADCC assay**

LK2 tumors (~14 mm^3) were established in Foxn1nu mice (four tumor sites per mouse) and were treated with PEP005 as described above. On day 11 after treatment initiation, antisera was collected by heart puncture and pooled. A parallel group of mice were not treated and sera was also collected on day 11 after treatment initiation, antisera was collected by heart puncture and pooled. A parallel group of mice were not treated and sera was also collected on day 11, by which time the tumors had grown to 30–60 mm^3. Sera from naive animals was taken at the same time (n = 3/group). LK2 cells were seeded in triplicate (4×10^3/well) in 96 round-bottom wells and were incubated for 2 h at 4°C with a 1/3 dilution of the antisera (total volume, 13 μl). Guinea pig complement (Invitrogen Life Technologies) (final dilution 1/10), PEP005 (final concentration 10 ng/ml) or medium (control) was then added with murine neutrophils at an E:T ratio of 100:1 (final total volume 150 μl). The plates were kept at 4°C and spun at 50 g for 5 min. The plates were then incubated at 37°C for 4 days with two medium changes, and total cellular protein of the adherent LK2 cells was then measured using crystal violet staining as described previously (28). Murine neutrophils were prepared as described previously (29) except that mice received two i.p. injections of casein separated by 18 h before peritoneal lavage, and the RBC lysis step was omitted.

**Results**

**Neutrophil recruitment and tissue morphology after PEP005 treatment**

The UV-induced murine squamous cell carcinoma line LK2 was grown as s.c. tumors in Foxn1nu mice. H&E staining of tumor sites and normal skin 24 h after topical treatment with placebo (isopropanol gel) revealed normal morphology with small numbers of leukocytes present (Fig. 1, A–D). Skin obtained 6 h after topical application of PEP005 showed decreased integrity of hair follicles and sebaceous glands, as well as increased dilation of local blood vessels. An increase in neutrophil numbers was observed in the treated area (Fig. 1, E and F). Similar results were also seen in the PEP005-treated tumor sites at this time (Fig. 1, G and H). Twenty-four hours after treatment with PEP005, a large number of neutrophils and some macrophages were found in treated skin (Fig. 1, I and J), and in and around the treated tumor site, with neutrophils abundant at the periphery of the tumor. Neutrophils can be seen as the small spherical and uniform cells with red/pink cytoplasm and irregular blue nuclei. Areas with a pronounced neutrophil infiltrate are encircled (Fig. 1 F, H, J, and L). Some hemorrhage of local blood vessels was also apparent at this time (Fig. 1, K and L). High magnification images of areas at the periphery of the tumor following placebo treatment show intact tumor cells, RBC, and few leukocytes (Fig. 1M). In contrast, high magnification images of areas at the periphery of the tumor following PEP005 treatment

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**FIGURE 1.** Leukocyte recruitment and tissue morphology after PEP005 treatment of normal skin and tumor sites. LK2 tumors (~14 mm^3) (two tumor sites per mouse and two mice per group) growing on the flanks of Foxn1nu mice, and two normal skin sites on the opposite flanks were treated once topically with PEP005 or placebo. Representative photomicrographs are shown of the histology of normal skin 24 h after treatment with placebo (isopropanol-based gel) (A and B), 6 h after treatment with PEP005 (in isopropanol-based gel) (E and F) or 24 h after PEP005 treatment (I and J). Representative photomicrographs are also shown of the histology of LK2 tumor sites treated with placebo (C and D), 6 h after treatment with PEP005 (G and H) or 24 h after PEP005 treatment (K and L). The tumor can be readily seen as the large spherical mass at the centers of panels C, G, and K. Areas containing a pronounced neutrophil infiltrate are encircled. Bar represents 0.25 mm for A, C, E, G, I, and K (low magnification) and 0.015 mm for B, D, F, H, J, and L (medium magnification). High magnification images of tumor sites 24 h after treatment with placebo (M) and PEP005 (N and O) are also shown. Bar represents 30 μM.
show numerous neutrophils, which can be identified by their multilobed nuclei and red staining nongranular cytoplasm (Fig. 1, N and O). A similar pattern of neutrophil infiltration was also observed when this experiment was repeated using C57BL/6 mice and B16 melanomas (data not shown). The acute inflammatory response that follows PEP005 treatment was therefore characterized by a pronounced neutrophil infiltrate.

As reported previously, the cosmetic effect after PEP005 treatment was very favorable. Approximately 3 wk after PEP005 treatment, the skin at the treatment site was similar to untreated skin and had normal elasticity, and by 2–3 mo, few if any signs of scarring or erythema were apparent (12).

Tumor relapse in neutrophil-depleted mice after topical chemotherapy with PEP005

We have previously shown that PEP005 was able to cure, without significant relapse, a number of different tumor types grown in Foxn1nu mice, illustrating that T cells are not required for effective treatment (12). Neutrophils are fully active in Foxn1nu mice and their importance for the anticancer activity of PEP005 was investigated by using the anti-Ly-6G Ab (RB6-8C5) to deplete these cells in mice bearing LK2 tumors. The tumors grew only marginally slower in control animals (that had received no Ab) and animals receiving control Ab (A95-1), compared with animals given the anti-Ly-6G (RB6-8C5) Ab (Fig. 2A).

In a separate experiment, LK2 tumors were established in control animals, animals receiving control Ab and animals receiving anti-Ly-6G Ab. When the tumors had reached ~14 mm3 they were treated topically with PEP005 daily for 3 days. Initial chemoablation of the tumors was apparent in all groups; however, after day 25 in the anti-Ly-6G Ab-treated group tumors began to re-emerge (Fig. 2B). Data from this experiment are also presented as a percentage of tumors relapsing in each group over time (Fig. 2C). In control animals, only 1 of 12 (8.3%) tumors relapsed and none of the tumors relapsed in animals that had received the control Ab (Fig. 2C). However, in animals whose neutrophils had been depleted with anti-Ly-6G Ab (see below), a significant increase in the relapse rate to 83% was observed (Fig. 2C) (Cox regression analysis p = 0.005, Wald statistic = 7.92, for anti-Ly-6G Ab vs control Ab). These data suggest that neutrophils are required to prevent relapse following PEP005 treatment.

Together with the PEP005-treated animals described in Fig. 2, B and C, a parallel group of animals was established that was treated with placebo (instead of PEP005). Acute erythema was absent in all placebo-treated animals, but apparent in all PEP005-treated tumor sites (Fig. 2D). However, the peak erythema on day 2 was ~50% lower in the mice that received the anti-Ly-6G Ab compared with control Ab-treated animals (p < 0.001, unpaired Student’s t test) (Fig. 2D). The neutrophil infiltrate thus appeared to contribute significantly to the PEP005-induced inflammatory response.

The neutrophil counts in blood were determined for each of the groups shown in Fig. 2. The white cell counts in placebo-treated mice (Fig. 3A), mice treated with PEP005 (Fig. 3B), and mice treated with the control Ab (data not shown) were essentially identical. In contrast, the percentage of neutrophils in the blood fell dramatically from ~80% of total leukocytes to ~3% following anti-Ly-6G Ab treatment in both placebo-treated and PEP005-treated animals (Fig. 3, C and D). Neutrophil counts were restored to normal levels ~10–12 days after the last Ab injection (in Fig. 3, C and D, the increase in the percentage of lymphocytes over days 0–10 did not reflect an increase in the number of lymphocytes per microliter of blood; data not shown).

**FIGURE 2.** The effect of granulocyte depletion on PEP005 treatment of LK2 tumors grown on Foxn1nu mice. A, Tumor growth in control mice and animals given anti-Ly-6G Ab. LK2 tumors were injected s.c. (n = 12 tumors/group, 4 tumors/mouse, 3 mice/group) on day −14. Mice were injected with anti-Ly-6G Ab (100 μg i.p., on days −2, 0, and 2) (●), or with control Ab (A95–1; 100 μg i.p, on days −2, 0, and 2) (○) or with nothing (□). Tumors were treated with placebo on days 0, 1, and 2. The tumor volumes represent the mean volume of individual tumors. Hash symbols indicate data points where one or more of the mice were euthanized (due to excessive tumor burden), with the means including the tumor volume(s) observed at euthanasia. B, Tumor growth in control mice and animals given anti-Ly-6G Ab after PEP005 treatment. As for A except LK2 tumors (~14 mm3) were treated with 10 μg of PEP005 in isopropyl-based gel daily for 3 days on days 0, 1, and 2 (symbol legend and hash symbols as for A). C, Percentage of tumor sites relapsing in control mice and animals given anti-Ly-6G Ab after PEP005 treatment. Data from the experiment described in B showing the percentage of tumor sites relapsing over time after PEP005 treatment (100% = 12 tumor sites) (symbol legend as for A). The tumors were deemed to have relapsed when they had reached 8 mm3 (the first tumor to relapse was on mouse 1, the next on mouse 3, then mouse 2, 2, 3, 2, 3, 1, 2, and 1, illustrating that relapse rates were not different for individual mice and that each tumor could be considered as an independent entity). D, Erythema of tumor sites in control mice and animals given anti-Ly-6G Ab after PEP005 or placebo treatment. The erythema area represents the mean of the areas of erythema at the treatment sites (n = 12/group). Symbol legend as in A plus placebo-treated mice after 1) anti-Ly-6G Ab administration (□), 2) after control Ab administration (●), and 3) after no Ab administration (△). The experiments described in this figure were repeated twice and one representative experiment is shown.
Rapid relapse after PEP005 treatment of B16 tumors in CD18-deficient mice

Neutrophil extravasation into inflamed sites is severely impaired in CD18-deficient mice (23, 30, 31), whereas Ab and some T cell responses remain intact (32). B16 tumors grew faster in CD18-deficient mice compared with C57BL/6 mice (Fig. 4A), so PEP005 treatment was initiated earlier post-tumor cell implantation in the former so that tumor sizes were comparable in CD18-deficient and C57BL/6 mice. Although 100% of B16 tumors with an average volume of 14 mm$^3$ were cured with three topical applications of PEP005 in C57BL/6 mice, all tumors of a similar size treated in CD18-deficient mice rapidly relapsed after the same PEP005 treatment (Fig. 4A). An acute erythema was apparent after PEP005 treatment and the area was similar in CD18-deficient and wild-type mice, although the intensity was considerably less in the former animals. The erythema in CD18-deficient mice was mainly a discoloration with slight reddening seen on some mice, whereas wild-type mice showed pronounced reddening in all treated sites (data not shown).

Thus, in a second neutrophil-defective model, PEP005-treated tumors relapsed and the intensity of the PEP005-induced inflammation was reduced. Although multiple leukocyte interactions can be affected by CD18 deficiency, these data support the view that an acute inflammatory response with a dominant neutrophil infiltrate is required for the antitumor efficacy of PEP005.

FIGURE 3. Blood leukocyte counts in mice given anti-Ly-6G and control Abs. Tail vein blood was taken from the mice described in Fig. 2 and smeared onto glass slides. White cell counts were performed by microscopy after Quick Dip staining (cells on two slides were counted per time point). The number of individual cell types is expressed as a percentage of total white cells (± SE); neutrophils (□), lymphocytes (△), monocytes (●), and eosinophils (▲). A, Control mice treated with placebo (as in Fig. 2A). B, Control mice treated with PEP005 (as in Fig. 2B). C, Mice injected with anti-Ly-6G Ab and treated with placebo (as in Fig. 2A). D, Mice injected with anti-Ly-6G Ab and treated with PEP005 (as in Fig. 2B).

FIGURE 4. Relapse rates following PEP005 treatment in CD18-deficient, NK-depleted, op/op, and SCID mice. A, Rapid relapse of B16 melanoma in CD18-deficient mice. B16 cells were injected s.c. on the back of B6.129S7-Itgb2tm1Bay/J CD18 hypomorphic mice. Six days later when tumors had reached ~14 mm$^3$, the animals were treated daily for 3 days (days 0, 1, and 2) with placebo (●) or 10 µg of PEP005 (▲) (n = 9 tumors/group, 1 tumor/mouse). B16 cells were implanted s.c. on the backs of C57BL/6 mice and were treated, 7 days after inoculation (when the tumors had reached ~14 mm$^3$), daily for 3 days with placebo (□) or 10 µg of PEP005 (▲) (n = 8 mice/group, 1 tumor/mouse). The indicated tumor volumes represent the mean volume of individual tumors (± SE). B, The role of NK cells. Percentage of LK2 tumor sites relapsing in control Foxn1nu mice (◆) and Foxn1nu mice given anti-asialo GM1 Ab (□) after PEP005-treatment of ~14 mm$^3$ tumors (n = 12 tumors/group, 4 tumors/mouse). C, The role of macrophages. B16 tumor growth in op/op mice following treatment with placebo (□) or 10 µg of PEP005 (▲) (n = 12 tumors/group, 4 tumors/mouse; 100% = 12 tumor sites). D, Relapse of LK2 tumors following PEP005 treatment in SCID mice. LK2 tumors were injected s.c. into SCID and Foxn1nu mice (n = 12 tumors/group, 4 tumors/mouse; 100% = 12 tumor sites). After 2 wk when tumors had reached ~14 mm$^3$, the tumors were treated with PEP005 as above and relapse rates monitored. Individual placebo-treated tumors reached an average size of 270 mm$^3$ by day 28 in both SCID and Foxn1nu mice (data not shown). The experiments in this figure were performed once.
The role of NK cells and macrophages

NK cells and macrophages are present in Foxn1
mice and these cell types can be activated by PKC-activating agents (33, 34). To determine their potential contribution to the antitumor effects of PEP005, the experiment shown in Fig. 2 was repeated using a polyclonal anti-asialo GM1 polyclonal Ab to deplete NK cells (tumor cells grown between 1.5 and 2 times faster in NK-depleted animals compared with controls; data not shown). The erythema (data not shown) and relapse rates were not significantly different between these groups (Fig. 4B) (p = 0.47). Thus, NK cells did not appear to have a significant role in preventing relapse after PEP005 treatment.

The experiment using B16 tumors shown in Fig. 4A was repeated in Csfm
/Csfm
(op/op) mice, which lack functional macrophage-CSF, and are therefore severely monocyteopenic (35). PEP005 treatment was able to cure, without relapse, 100% of tumors (Fig. 4C), indicating that macrophages do not have an important role in preventing relapse following PEP005 treatment.

Relapse of PEP005-treated tumors in SCID mice

To determine whether successful relapse free PEP005 treatment requires B cells and Ab production, LK2 tumors were grown in SCID mice and were treated with PEP005 and monitored for relapse. The relapse rate of PEP005-treated LK2 tumors grown in SCID mice (Fig. 4D) was very similar to that seen for PEP005-treated LK2 tumors grown in granulocyte-depleted Foxn1
mice (Fig. 2C), with >80% of tumors relapsing by day 40. In contrast, nearly all the tumors were cured when similar sized LK2 tumors grown in Foxn1
mice were treated with PEP005 (Cox regression analysis, p = 0.006, Wald statistic = 7.68), for relapse rates in SCID compared with Foxn1
mice. LK2 tumors grew at similar rates in SCID and Foxn1
mice and the PEP005-induced erythema was similar in the two mouse strains (data not shown). The high relapse rates in SCID mice indicates that anti-cancer Ab production following PEP005 treatment is also required to prevent relapse.

Induction of MIP-2, TNF-α, and IL-1β after PEP005 treatment in vivo.

To determine whether the rapid inflammation induced by PEP005 was associated with production of proinflammatory mediators, RT-PCR was used to assess IL-6, KC/Groα, MIP-2, TNF-α, and IL-1β mRNA expression in PEP005-treated tumor sites and PEP005-treated normal skin. mRNA isolated from full thickness mouse skin that had been treated with PEP005 showed a ~250-fold increase in MIP-2 mRNA, a ~8-fold increase in TNF-α and a ~2-fold increase in IL-1β mRNA within 6 h of treatment (Fig. 5A). Tumor tissue was separated from surrounding skin and dermal tissue and showed a ~17-fold increase in MIP-2 mRNA, a ~2-fold increase in TNF-α, and a ~1.5-fold increase in IL-1β mRNA within 6 h of treatment (Fig. 5B). No changes in IL-6 and KC/Groα transcripts were detected (data not shown). Thus, in both skin and tumor tissue in vivo PEP005 treatment induced MIP-2, TNF-α, and to a lesser extent IL-1β, all mediators involved in neutrophil migration and activation (15, 36).

Proinflammatory cytokine induction in human keratinocytes, fibroblasts, neutrophils, and melanoma cells after PEP005 treatment in vitro

To determine whether the inflammatory mediator profile induced in mice following PEP005 treatment also applies to human cells, the induction of cytokines and chemokines following PEP005 treatment of human keratinocytes, neutrophils, and a human melanoma cell line (Me10538) was assessed in vitro. Culture medium was analyzed for cytokines and chemokines 6 h after treatment with PEP005. IL-8, the human counterpart of MIP-2, was induced in all cells tested, with keratinocytes and neutrophils producing maximal levels at 5 ng/ml PEP005 (Table I). The

Table I. Induction of proinflammatory cytokines in human cells in vitro

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<th>PEP005 (ng/ml)</th>
<th>Keratinocytes</th>
<th>Fibroblasts</th>
<th>Melanoma</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-8</td>
<td>TNF-α</td>
<td>IL-6</td>
<td>IL-8</td>
</tr>
<tr>
<td>0</td>
<td>995 ± 48</td>
<td>8 ± 1</td>
<td>ND</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>3910 ± 148</td>
<td>510 ± 26</td>
<td>ND</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>5</td>
<td>4775 ± 178</td>
<td>847 ± 37</td>
<td>ND</td>
<td>160 ± 14</td>
</tr>
<tr>
<td>10</td>
<td>3895 ± 198</td>
<td>498 ± 29</td>
<td>ND</td>
<td>215 ± 12</td>
</tr>
<tr>
<td>100</td>
<td>2950 ± 108</td>
<td>335 ± 21</td>
<td>ND</td>
<td>239 ± 9</td>
</tr>
</tbody>
</table>

* Cells (10⁶ cells per well for neutrophils and 5 x 10⁴ cells per well for the other cells) were incubated with the indicated concentration of PEP005 for 6 h and the supernatants were analyzed in triplicate for the indicated cytokines. ND, Not detectable.
reduced response of neutrophils at 100 ng/ml PEP005 (compared with 5 ng/ml) was probably due to apoptosis, as PEP005 was a potent inducer of neutrophil activation (see below), which leads to activation-induced cell death. TNF-α levels were induced 60-fold in keratinocytes in the presence of 1 ng/ml PEP005, and IL-6 was marginally induced in fibroblasts after treatment with >10 ng/ml PEP005 (Table I). No IL-2, IL-4, IL-10, IL-12, or GM-CSF was detected in any cell type tested after PEP005 treatment (data not shown). Thus, PEP005 induced production of the neutrophil chemoattractant IL-8 in keratinocytes, fibroblasts, neutrophils, and tumor cells, and potently induced TNF-α production in keratinocytes.

**Activation of human endothelial cells by PEP005**

Recruitment of neutrophils to the site of inflammation requires activation of the vascular endothelium to promote neutrophil binding, which is a prerequisite for extravasation and tissue infiltration (37). PEP005 was able to activate vascular endothelial cells in a dose-dependent manner with significant neutrophil binding occurring at 10 ng/ml PEP005 (Fig. 6A). TNF-α is known to activate vascular endothelium; neutrophil binding induced by TNF-α was shown as a positive control (Fig. 6A).

**Induction of tumoricidal reactive oxygen species by PEP005-activated human neutrophils**

The production of potential antitumor agents by PEP005-stimulated human neutrophils was investigated. PEP005 concentrations >10 ng/ml were able to induce marked superoxide production by human neutrophils (Fig. 6B). At 100 ng/ml (but not 10 ng/ml), PEP005 induced a modest release of defensins (data not shown) which are neutrophil granule proteins that have been reported to have some anticancer activity (38). Soluble TRAIL (Apo-2 ligand)
(39) production was not induced at PEP005 concentrations of 1–100 ng/ml (data not shown). Thus, PEP005 by itself did not efficiently induce neutrophil degranulation, whereas PEP005 was able to induce generation of reactive oxygen species.

MM96L human melanoma cells are known to be sensitive to reactive oxygen species (40) and these mediators when secreted by neutrophils can be cytotoxic for tumor cells (2, 41). To determine whether PEP005-induced reactive oxygen species could mediate antitumor activity, human neutrophils were cocultured with MM96L cells in the presence and absence of PEP005 (10 ng/ml). Neutrophils and drug were removed after 24 h and the cells cultured for a further 6 days. In the absence of PEP005, the neutrophils had no effect on the viability of MM96L cells. However, in the presence of PEP005, the neutrophils were able to reduce the viability of MM96L cells by \( \sim 50\% \) at a neutrophil to target ratio of 3:1 and \( \sim 90\% \) at a neutrophil to target ratio of 100:1 (Fig. 6C).

**Increased anti-cancer Ab levels after PEP005 treatment of tumors**

Adaptively transferred anti-tumor Abs are known to promote neutrophil-mediated ADCC of tumor cells (2, 6, 7). To determine whether PEP005 treatment leads to increased anti-tumor Ab levels, B16 tumors growing in C57BL/6 mice were treated with PEP005. As early as day 11 post-PEP005 treatment of \( \sim 14 \)-mm\(^3\) tumors, anti-cancer Ab levels were elevated, with responses significantly exceeding those seen in untreated animals that had large tumor burdens (\( \sim 30–60 \) mm\(^3\)) at this time point (Fig. 6D) (\( p = 0.045 \) at 1/100 dilution, unpaired Student’s t test). Thus, PEP005 treatment of tumors appears to increase the levels of anti-cancer Abs.

**Murine neutrophil-mediated ADCC of LK2 cells in vitro**

The data from Figs. 2C, 4D, and 6D suggest that tumor relapse following PEP005 treatment of LK2 tumors on Foxn1\(^{nu}\) mice is prevented by neutrophil-mediated ADCC. Foxn1\(^{nu}\) mice cannot generate significant IgG responses, but they do generate IgM responses that can fix complement and thereby trigger neutrophil degranulation via complement receptors (42). We thus sought to determine whether sera from Foxn1\(^{nu}\) mice, whose LK2 tumors had been treated with PEP005, would be able to mediate ADCC in vitro. Antiserum from such mice was able to reduce significantly (\( p = 0.006 \), unpaired Student’s t test) the viability of LK2 cells in the presence of murine neutrophils and added complement (Fig. 6E, +Complement, PEP005 LK2). Neither antiserum from Foxn1\(^{nu}\) mice, whose LK2 tumors had not been treated, nor antiserum from naive Foxn1\(^{nu}\) mice, was able to reduce significantly the viability of LK2 cells under the same conditions (Fig. 6E, +Complement, LK2, and Naive). The lower levels of complement present in the mouse serum (which was not heat inactivated) were insufficient under these assay conditions to activate the antitumor activity of the neutrophils (Fig. 6E, Controls). However, when 10 ng/ml PEP005 was added, antitumor activity was again seen for the PEP005 LK2 antiserum, but not the LK2 or naive serum (Fig. 6E, +10 ng/ml PEP005). In the absence of PEP005 or added complement, none of the antisera were able to reduce significantly the viability of LK2 cells (Fig. 6E, Controls). Thus, antiserum from Foxn1\(^{nu}\) mice, whose LK2 tumors had been treated with PEP005, was able to induce neutrophil-mediated ADCC of LK2 cells in vitro in the presence of added complement or low levels of PEP005.

**Discussion**

Neutrophils have long been recognized as key players in innate defenses against microbial infections, but recently insights into their potential roles in anticancer activity have begun to emerge (1–6, 9, 10). Herein, evidence is provided that neutrophil-mediated elimination of residual tumor cells prevents tumor relapse after topical treatment with the novel anticancer drug, ingenol-3-angelate (PEP005). Initially, PEP005 treatment results in direct cytotoxic activity against skin tumors (12) and this is followed by an acute inflammatory response in which neutrophils form the bulk of the cellular infiltrate. We show that ablation of neutrophil activity in two murine tumor models resulted in reduced inflammation and significant increases in tumor relapse rates following PEP005 treatment. PEP005 induced MIP-2/IL-8, TNF-\( \alpha \) and IL-1\( \beta \); all mediators involved in neutrophil recruitment and/or activation (2, 15, 36). PEP005 also activated endothelial cells resulting in neutrophil binding, a prerequisite for neutrophil extravasation (37). PEP005 treatment resulted in increased anti-tumor Ab responses and a high relapse rate was observed in SCID mice following PEP005 treatment. Antiserum, from mice whose LK2 tumors had been treated with PEP005, was also able in the presence of murine neutrophils and complement or PEP005, to reduce the viability of LK2 cells in vitro. In addition, PEP005 induced the release of tumorcidal reactive oxygen species in human neutrophils. PEP005 treatment thus appears to have a two stage mechanism of action, initial chemotaxis of neutrophils followed by a neutrophil-mediated eradication of residual tumor cells by ADCC.

The initial cytotoxic activity of PEP005 involves rapid disruption of the plasma membrane, rapid swelling of mitochondria and death by primary necrosis. The process does not appear to require PKC activation and occurs at PEP005 concentrations of \( \sim 100 \) \( \mu \)g/ml (12). At lower concentrations (10–100 ng/ml), PEP005 is a potent activator of PKC (13). This level of PEP005 is probably reached within a day or two around the tumor site due to drug dispersal and conversion of PEP005 to inactive products, which in tissue culture occurs with a half life of \( \sim 5–10 \) h (data not shown).

Application of the PKC activator 12-0-12-tetradecanoyl-phorbol-13-acetate (TPA) to mouse skin also induces a pronounced T cell-independent influx of neutrophils (14, 15, 43). TPA is also able to stimulate neutrophils to produce reactive oxygen intermediates (44). The activation of PKC by PEP005 is therefore likely to be responsible for the PEP005-mediated recruitment and activation of neutrophils.

PEP005 treatment induced MIP-2/IL-8, TNF-\( \alpha \), and IL-1\( \beta \), all mediators that would contribute to the recruitment and activation of neutrophils (2, 15, 36). In vivo neutrophil extravasation requires activation of the endothelium (37). This process is induced by TNF-\( \alpha \) and IL-8, which increase the affinity of integrins on neutrophils and their adhesion partners on the endothelial cells (45, 46). PEP005 was also able to activate endothelial cells directly, perhaps providing a second rapid and direct boost to neutrophil recruitment. MIP-2/IL-8 showed the greatest increase in response to PEP005 and previous studies have demonstrated that Foxn1\(^{nu}\) mice challenged with human tumor cells expressing IL-8 showed extensive neutrophil influx to the tumor site resulting in dramatically reduced tumor growth (47). The release of MIP-2/IL-8 by tumor and stromal cells may therefore represent a key immunotherapeutic aspect of PEP005 action, allowing it to harness the anticancer potential of neutrophils.

The high relapse rates of PEP005-treated tumors grown in (B cell defective) SCID mice, the increase in anti-cancer Abs following PEP005 treatment, and the ability of antiserum from mice, whose tumors had been treated with PEP005, to mediate neutrophil ADCC in vitro, indicate that once recruited to the tumor site, killing of residual cancer cells by neutrophils involves ADCC. This mechanism has recently been shown to be involved in the anticancer activity of adoptively transferred anti-cancer Abs (5–7). How PEP005 treatment might lead to increased anti-cancer Ab...
production remains unclear. However, the induction of primary necrosis of the tumor cells by PEP005 treatment (12) may be involved because primary necrosis has been shown to be immunostimulatory (48, 49). PKC activation (13) may also promote denritic cell (50) and/or B cell maturation (51). In vitro, PEP005 was able to induce release of tumoricidal reactive oxygen intermediates. Furthermore, like TPA (52), PEP005 was unable to stimulate efficient degranulation of human neutrophils. However, PKC stimulation and FcR engagement have been shown to act synergistically on neutrophils to stimulate degranulation and killing of tumor cells (52), a synergy that also appears to apply to PKC stimulation and complement receptor engagement (Fig. 6E, ±10 ng/ml PEP005). PEP005 thus appears to provide an environment that is highly conducive to neutrophil-mediated anticancer activity. Neutrophils are recruited and activated by PEP005-induced inflammatory mediators and are also activated by the direct action of PEP005. The PEP005-induced anti-cancer Abs then provide tumor cell targeting and Fc and/or complement receptor-mediated triggering.

In summary, our data show that neutrophil-mediated killing of residual tumor cells is important for preventing relapse following PEP005 treatment of skin cancers. This study also provides additional evidence that in specific settings neutrophils can provide important anticancer activity.

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Disclosures

S. M. Ogbourne is now an employee of Peplin Ltd, but was not when the work was being performed. A. Suhrbier and P. G. Parsons are consultants for Peplin Ltd.

References


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