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Advances in Brief

Role of Interleukin 1 and Granulocyte Colony-Stimulating Factor in Photofrin-based Photodynamic Therapy of Rat Rhabdomyosarcoma Tumors[1]

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Abstract

Neutrophils play an important role in the efficacy of photodynamic therapy (PDT). These leukocytes rapidly accumulate into the tumor lesion after PDT and most likely eradicate the remaining attenuated tumor cells. The underlying mechanism of the accumulation of neutrophils at the time of PDT is not known. Therefore, we determined the effect of PDT on the course of mature and immature neutrophils in the circulation of rhabdomyosarcoma-bearing rats and studied the changes in the level of interleukin (IL)-1β as an important stimulator of the proliferation of precursor cells of the granulocyte lineage in the bone marrow.

We found that the effect of PDT on tumor growth was preceded by a rapid and specific increase of the number of mature neutrophils in the peripheral blood as early as 4 h after the start of PDT treatment and reaching maximum values after 8 h. At 24 h, the neutrophil numbers in the PDT-treated rats were still elevated as compared to sham-treated rats. In sham-treated rats, the numbers of blood monocytes and lymphocytes decreased by about 50% after 2 h and returned to their normal levels as soon as 2 h later. In PDT-treated rats, the course of monocyte numbers showed a similar pattern; however, lymphocyte numbers did not reach the normal range until 24 h.

The specific increment of neutrophils was preceded by an increase of band neutrophil numbers and elevated serum levels of IL-1β which were maximal at 2 h after the start of PDT. Pearson correlation analysis showed a significant association between the serum levels of IL-1β at this time point and the number of band neutrophils at 4 h (R^2 = 0.58; P = 0.03) and the number of mature neutrophils at 8 h (R^2 = 0.54; P = 0.04). This suggests that PDT evoked an IL-1-dependent increased production rate of neutrophils in the bone marrow. Further investigation showed that the injection of anti-granulocyte colony-stimulating factor (G-CSF) antibodies not only attenuated the increase in neutrophil numbers but also greatly decreased the efficacy of PDT. On this basis, we suppose that an IL-1-induced release of G-CSF by PDT underlies this nonspecific immune reaction to the tumor. Apparently, G-CSF not only stimulates the production rate of neutrophils in the bone marrow but also increases the functional activity of these leukocytes to become indispensable tumor cell killers.

Introduction

Increasing evidence arises that besides direct tumor cell kill due to the formation of singlet oxygen (1) and indirect tumor cell kill as a result of deprivation of oxygen and nutrients due to vaso-occlusion (2), PDT[3] also elicits a nonspecific (3, 4) and a specific immune response directed to the tumor (5). A central role in this immune response might be performed by the endothelial cells, which contract at the time of PDT, as has been observed *in vivo* (6) and studied *in vitro* (7), exposing the thrombogenic subendothelium. Besides the fact that this endothelial contraction stimulates edema formation, platelet aggregation, thromboxane release, and thrombus formation, which eventually leads to blood flow stasis (8, 9), it also will facilitate the extravasation of blood leukocytes into the underlying tumor tissue.

Within 5 min after the start of PDT, neutrophilic granulocytes adhere to the vascular wall (10) and infiltrate the tumor area (11), where they may kill the attenuated tumor cells directly (12) or via a complex interaction with other cells (13). The concept that leukocytes may play an important role in the PDT-induced inflammatory reaction has led to several immunotherapeutic approaches to enhance the efficacy of PDT (4, 14–16). These strategies indeed potentiated the effect of PDT, stressing the important role of a cellular inflammatory reaction in tumor kill.

Because we have shown recently that an effective interstitial PDT of rhabdomyosarcoma tumors is dependent on the presence of neutrophils in the circulation (3), the question was raised whether PDT by itself influences the number of blood neutrophils. In the present study, therefore, we established the effect of PDT on the course of neutrophils in the circulation of rats bearing rhabdomyosarcoma R-1 tumors and examined the underlying regulatory mechanism.

Materials and Methods

Animals and Tumor Model. Female WAG/Rij rats, aged 10–15 weeks, were obtained from Harlan (Zeist, the Netherlands). Small pieces of a well-characterized isologous rhabdomyosarcoma, designated as R-1 (17), were implanted s.c. into the thighs. Tumor growth was assessed by caliper measurements on three orthogonal diameters, once a day. Tumors were treated when the volume was between 750 and 1500 mm^3. Blood samples were obtained by tail bleeding with EDTA as the anticoagulant. Total numbers of leukocytes were determined with a microcell counter. Differential counts were performed on May-Grünwald and Giemsa-stained blood smears in quadruplicate.

Photosensitizer and Light Delivery. The photosensitizer PII was obtained from Quadra Logic Technologies, Inc. (Vancouver, British Columbia, Canada) and was reconstituted in 5% glucose before i.v. administration into a tail vein at 10 mg of PII/kg 24 h prior to light delivery.

The light source was a dye laser pumped by an Argon ion laser (Spectra Physics models 375B and 2040E, respectively). A birefringent filter and monochromator were used to tune the dye laser to emit light at 625 ± 1 nm wavelength using 4-dicyanomethylene-2-methyl-6(*P*-dimethylaminostytyl)-4*H*-pyran as a dye. The light was directed via a beam splitter to three cylindrical diffusers of a 15-mm length (Rare Earth Medical, Dennis, MA).

Treatment Protocol. Interstitial PDT treatment was performed as described previously (3) with minor adaptations. In brief, rats were anesthetized by i.m. injection of 1 ml/kg Hypnorm (fluanisol/fentanyl mixture; Janssen Pharmaceutica, Beerse, Belgium). Animals were placed on a heated support during treatment to control body temperature. A diffusor was inserted into the central axis of the tumor parallel to the body axis. The output of the diffuser was kept below 50 mW per cm of diffuser length to avoid hyperthermia. Tumor core temperature was measured during PDT using a thermocouple probe and never exceeded 40°C. Total applied radiant energy was 270 J per cm of diffusor length. Control rats were treated in the same way as above except for the illumination protocol (*i.e.*, PII injection only) or PII injection (*i.e.*, illumination only).

For studies on the involvement of growth factors, rats were injected with 500 μg of sheep polyclonal antibody against recombinant human G-CSF

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[3] The abbreviations used are: PDT, photodynamic therapy; PII, Photofrin II; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor.

(Biodesign International, Kennebunk, ME) i.v. 1 h prior and i.p. 8 h after the start of PDT treatment.

Cytokine ELISA. IL1-β and TNF-α levels in sera were determined by rat-specific ELISA kits obtained from Biosource Europe S.A. (Fleurus, Belgium).

Statistical Analysis. Data were analyzed by paired or unpaired Student's *t* test and by Pearson correlation analysis where appropriate and considered significant when $P < 0.05$.

Results

Effect of PDT on Tumor Growth. To study the effect of PDT on the number of neutrophils in the circulation, we evaluated the efficacy of the applied PDT treatment on the growth of rhabdomyosarcoma tumors first. As shown in Fig. 1, tumor growth of rats treated with PII or illumination only was not affected, whereas PDT treatment did affect tumor growth. Strikingly, 1 day after PDT treatment, the tumor volumes were significantly increased as compared to illumination ($P = 0.025$) or PII ($P = 0.016$) only. However, at 2 days after PDT treatment, the tumor volumes were decreased to approximately the original volume at the start of treatment. At this point, tumor growth after PDT treatment was significantly retarded as compared to the control tumors. The growth delay (*i.e.*, the time that is needed for a tumor to reach its original treatment volume) was approximately 4.5 days. Thereafter, tumor growth resumed at the same rate as before the start of PDT.

Effect of PDT on the Numerical Course of Blood Leukocytes. After PDT or control treatment, the number of circulating lymphocytes (Fig. 2*A*) decreased at 2 h to about 50% of the original number at the start of treatment. Thereafter, their numbers increased again to approximately the normal level after 8 h for the control groups and after 24 h for the PDT treated group. The number of monocytes decreased also transiently to about 50% of the original value at 2 h after PDT or control treatment, reaching the pretreatment level 2 h later irrespective of the type of treatment (Fig. 2*B*). The number of neutrophils changed dramatically upon treatment (Fig. 2*C*). From 4 h after treatment onward, their number increased 4-fold in both control-groups to a maximum at 8 h after treatment, whereas PDT treatment even led to a 5-fold increase, which was significantly higher than the control

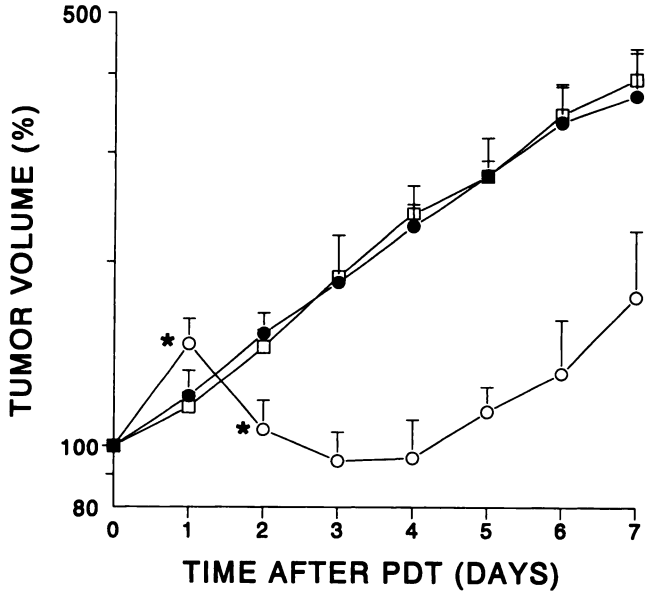

Fig. 1. Tumor growth of rhabdomyosarcoma after PDT. Volumes of PDT-treated tumors (○), of unilluminated tumors of rats injected with PII (●), and of illuminated tumors of rats without PII injection (□) are expressed as percentage of the tumor volume at the day of treatment. Data are the means of five rats; *bars*, SD. *, significantly different from control data $P < 0.05$, or see "Results" for the exact *P* value.

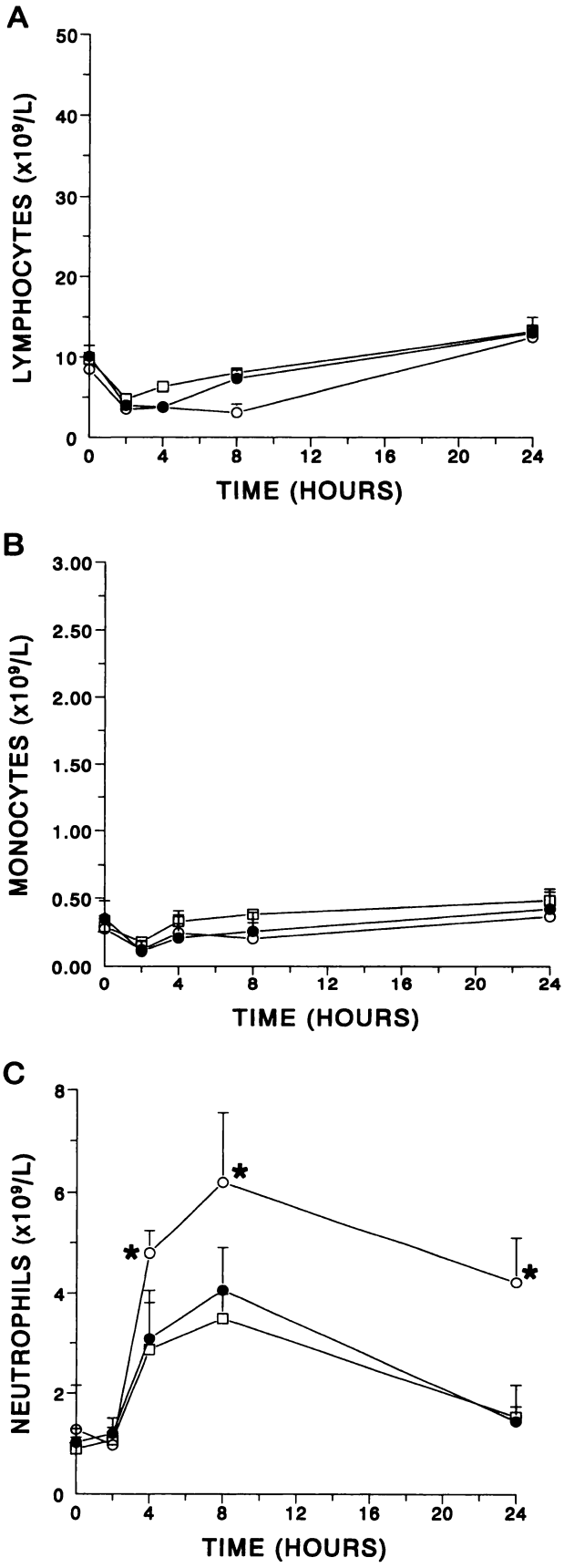

Fig. 2. Effect of treatment on the numbers of peripheral blood leukocytes. Shown are the numerical courses of lymphocytes (*A*), monocytes (*B*), and neutrophils (*C*) after treatment of rat rhabdomyosarcoma tumors by PDT (○), or injection with PII (●), or illumination only (□). Data are the means of five rats; *bars*, SD. *, significantly different from control data $P < 0.05$, or see "Results" for the exact *P* value.

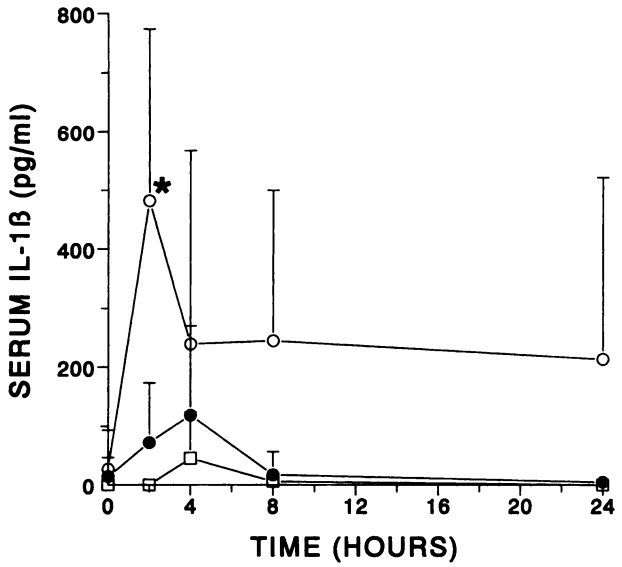

Fig. 3. Effect of PDT on serum IL-1β levels. Shown are the serum levels of IL-1β of rats treated by PDT (○), or injection with PII (●), or illumination only (□). Data are the means of five rats; bars, SD. *, significantly different from control data $P < 0.05$, or see "Results" for the exact P value.

groups ($P < 0.02$). At 24 h, neutrophil numbers were decreased to the normal level in the control rats, while being still elevated in the PDT-treated rats. Strikingly, immature band neutrophils appeared in the circulation as early as 2 h upon PDT treatment, reached a maximal number of $0.081 \pm 0.08 \times 10^9$/liter at 4 h after PDT, and subsided thereafter to below the detection limit (data not shown).

Effect of PDT on Serum Levels of IL-1β and TNF-α. To investigate the regulatory mechanism of the PDT-induced increase in the number of blood neutrophils, we determined the occurrence of the proinflammatory cytokines IL-1β and TNF-α in the sera of control and PDT-treated rats (Fig. 3). We found a transient but significant increase in IL-1 levels with a maximum at 2 h after PDT as compared to the level in control-treated animals ($P < 0.05$). Pearson correlation analysis showed a treatment-independent relationship between the serum level of IL-1 at 2 h and the number of band neutrophils at 4 h ($R^2 = 0.58$; $P = 0.03$) and the number of mature neutrophils at 8 h ($R^2 = 0.54$; $P = 0.04$). The levels of TNF-α were below the detection limit and did not change upon PDT or control treatment.

Involvement of G-CSF. To examine whether the increase in the level of IL-1β directly underlies the observed neutrophilia, we injected polyclonal anti-G-CSF antibody at time zero and at 8 h after the start of PDT. As shown in Fig. 4*A*, this antibody treatment attenuated the PDT-induced numerical increase in blood neutrophils. If expressed as the area-under-the-curve of blood neutrophils (dimension: $10^9 \times L^{-1} \times 24$ h) during the observation period of 24 h, these two consecutive injections of anti-G-CSF antibodies led to a decrease of about 25%, *i.e.*, from 118.6 to 87.1. In contrast to the moderate effect on the numerical course of neutrophils in the circulation, this anti-G-CSF treatment led to a dramatic decrease in the efficacy of PDT, amounting to an estimated delay of tumor growth of about 1 day as compared to 4.5 days under the normal condition (Fig. 4*B*).

Discussion

The major finding of this study was that interstitial PDT elicited a severe inflammatory reaction characterized by a profound neutrophilia that appeared dependent on an IL-1-induced release of G-CSF. Sham treatment of the tumor-bearing rats induced a (moderate) increase in the number of blood neutrophils as well, which was possibly evoked by tissue damage upon insertion of the optic fiber. However, this did not lead to retardation of tumor growth. Apparently, neutrophils are not able to kill those vital tumor cells directly. This confirms the results of a previous study showing that a direct effect of PDT on the tumor is necessary for neutrophils to become effective in tumor cell eradication, while in the absence of neutrophils, PDT in turn is also not effective (3).

It is noteworthy that the increase in the number of blood neutrophils after PDT most likely is an underestimation of the magnitude of the inflammatory reaction. Due to the migration and accumulation of neutrophils upon PDT into the tumor area (3, 11), the half-time of neutrophils in the circulation will decrease accordingly. Furthermore, by inducing the contraction of the endothelial cells lining the vessel wall, PDT treatment may also facilitate the accumulation of the neutrophils in the tumor. Indeed, exposure of the subendothelial matrix by PDT promotes the adhesion of neutrophils (7) and may also lead to edema formation, explaining the temporary increase of the tumor volumes at 1 day after PDT treatment (Fig. 1).

We found here that the peak in the number of mature blood neutrophils was preceded by a peak in the appearance of immature band neutrophils

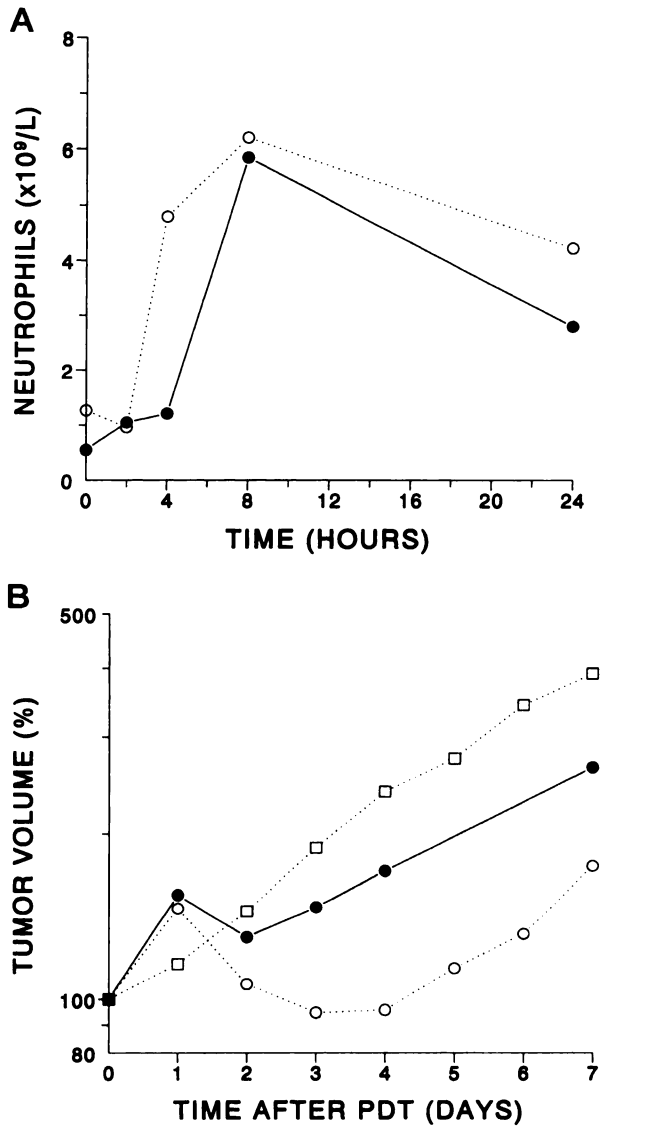

Fig. 4. Involvement of G-CSF in the PDT-induced neutrophilia. Shown are the courses of neutrophils in the circulation (*A*) and tumor volumes (*B*) of rats after PDT treatment (○; $n = 5$) or after PDT treatment in the presence of polyclonal anti-G-CSF antibody (●; $n = 2$). For comparison (*B*), the tumor volumes of rats after PDT treatment (○) and of rats injected with PII only (□; data of Fig. 1) are shown.

in the circulation, indicating that the production rate of neutrophils in the bone marrow is increased as well. This could be brought about by IL-1 that occurred in the circulation during the early phase after PDT. IL-1 is able to induce a granulocytosis by the mobilization of neutrophils from their storage pool in the bone marrow (18, 19). However, the results of the present study show that IL-1 does not act alone. The administration of anti-G-CSF antibodies revealed that the numerical increase of blood neutrophils is at least partially dependent on the endogenous production of the granulocyte-specific growth factor G-CSF. This growth factor stimulates the proliferation and maturation of neutrophils in the bone marrow and can also up-regulate the activation status of those leukocytes (20). It is conceivable that the release of G-CSF is triggered by IL-1β upon PDT. G-CSF can be produced by many cell types, including fibroblasts and endothelial cells after IL-1 stimulation both *in vitro* (21–23) and *in vivo* (24). In this respect, it is noteworthy that TNF-α is also capable of inducing G-CSF production as shown *in vitro* by endothelial cells (25) and *in vivo* (26). Macrophages release TNF-α following PDT treatment *in vitro* (27), but we were not able to demonstrate its occurrence in the circulation of the PDT-treated rhabdomyosarcoma rats.

Activated inflammatory cells make an essential contribution to the antitumor effect of PDT (3, 4, 11). Although G-CSF at least partially contributes to the neutrophilia, administration of anti-G-CSF antibodies has a much greater impact on the efficacy of PDT than could be deduced from its effect on the numerical course of blood neutrophils after PDT. This suggests that functional activation of neutrophils by the granulocyte-selective growth factor G-CSF is of great importance for the tumoricidal effect of PDT. It also supports the view that the local availability of this and other cytokines is essential to establish granulocyte-T lymphocyte cross-talk to overcome the immunosuppressive activity of the tumor (13). Neutrophils that can kill tumor cells upon activation by G-CSF (28) may also facilitate the establishment of a specific immune reaction against the tumor. Korbelik *et al.* (5) clearly showed that the activity of host lymphoid populations are essential for preventing the recurrence of EMT6 tumors in mice after PDT. In accordance with that study, we found a sustained decrease of the number of lymphocytes in the circulation during the first 24 h after PDT, suggesting their recruitment to the treated site.

Taken together, we suppose that the profound neutrophilia upon PDT of rhabdomyosarcoma-bearing rats is the result of the action of IL-1. However, IL-1 most likely did not act alone but conceivably stimulated the subsequent release of the granulocyte lineage-specific growth factor G-CSF. G-CSF in turn can increase the proliferation rate of myeloid precursors in the bone marrow but more importantly elevates the functional activity of neutrophils at the site of the tumor lesion. In the absence of G-CSF, neutrophils lack the ability to kill the attenuated tumor cells, making PDT much less effective. It is obvious that neutrophils are crucial for an effective PDT. Contraindications for PDT should, therefore, regard patients that are treated with severe immunosuppressive (cytostatic) agents unless recovered from granulocytopenia (by G-CSF). Future efforts in optimalization of PDT should concentrate on the leukocytes recognized to be important in the inflammatory reaction and the cytokines/growth factors important for their proliferation and activation.

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