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Release of clotting factors from photosensitized endothelial cells: a possible trigger for blood vessel occlusion by photodynamic therapy

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Photodynamic treatment of solid tumors results in the occlusion of blood vessels in the treated tissue. We hypothesize that this process is triggered by the release of one or more clotting factors from the photodamaged endothelial cells. Experimental evidence is presented that immediately after photodynamic treatment, cultured endothelial cells start releasing clotting factors into the medium in a dose range of minimal cytotoxicity.

Photodynamic therapy; Photosensitization; Phthalocyanine: Clotting factor: Endothelial cell

1. INTRODUCTION

Photodynamic therapy (PDT) is based on selective retention of photosensitizing dyes in the tumor tissue. Subsequent illumination with red light, usually from a dye laser, results in tumor necrosis with little damage to the surrounding normal tissue [1,2]. The necrosis most probably results from occlusion of the blood vessels [3–7]. The endothelium of blood vessels is quite sensitive to PDT [8] and plays an important role in the process of thrombus formation. Hence, it was reasonable to assume that the damage induced by photosensitization to endothelial cells causes the release of one or more clotting factors which trigger the process of thrombus formation and a subsequent blood flow stoppage.

Endothelial cells from bovine adrenal medulla that develop capillary-like growth patterns and produce factor VIII [9] and other clotting factors in culture [10] were chosen to test this hypothesis. Since it was recently shown that chloroaluminum phthalocyanine tetrasulfonate (ClAlPCS), a new and attractive sensitizer for PDT [11–13], is effective in stopping blood flow in experimental rat tumors [14], the release of clotting factors from these cells into the medium after photosensitization by ClAlPCS was investigated. The reason for performing this experiment was to understand some of the processes underlying photodynamic tumor destruction at the level of the blood supply to the tissue.

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2. EXPERIMENTAL

Endothelial cells from bovine adrenal medulla [9], line EJG-HBP, were grown in culture as a monolayer in minimal essential medium (MEM) supplemented with 10% fetal calf serum, 4 mM glutamine and antibiotics. The cells were harvested weekly and split at a ratio of 1:4. The cells doubled in number every 1.5 days at 37°C in a humidified atmosphere containing 5% CO_2. The cells were plated in 50 mm Petri dishes at a density of 2×10^5 cells per dish. 6 days after plating, when the cells reached confluency, ClAlPCS was added to the growth medium at a concentration of 4 μM. Exposure to light was performed after additional incubation for 18 h with the dye. The light source was a bank of 40 W fluorescent tubular lamps (Sylvania, Daylight) held in a reflector. The fluence rate was 55 $W \cdot m^{-2}$ at the surface of the cell monolayer.

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In the experiments with ^{51}Cr, following the incubation with the dye and $Na^{51}CrO_4$, and before exposure to light, the cells were rinsed three times with PBS and 2 ml Hank's balanced salt solution (HBSS) containing glucose was added. For radioactivity counting, samples of 1 ml of HBSS were transferred into counting vials together with 10 ml scintillation cocktail (Instagel). The remainder of the HBSS solution was discarded, the dishes were air-dried, the cells solubilized with 1 ml of hyamine hydroxide for 1 h at 37°C and radioactivity was counted in 10 ml toluene-based cocktail.

Clotting factors were assayed using the activated partial thromboplastin time (APTT) [15]. Briefly, 0.1 ml of factor VIII-deficient plasma was incubated at 37°C with 0.1 ml APTT reagent and 0.1 ml of cell extract. Following the incubation, 0.1 ml of 25 mM $CaCl_2$ was added to the reaction mixture and the time required to form a firm clot was measured using a coagulation timer. The clotting time of a test sample was converted to % clotting factor activity from a calibration curve obtained with normal human plasma. APTT reagent, normal human and factor VIII:C-deficient plasma were from Sigma Diagnostics.

3. RESULTS AND DISCUSSION

The effect of photosensitization by ClAlPCS on the growth of endothelial cells was studied over a range of light fluence values. As shown in fig.1, a low light dose (20 $kJ \cdot m^{-2}$) resulted in only a slight reduction in growth rate. However, following higher doses (e.g. 40 $kJ \cdot m^{-2}$) cell growth stopped completely, while at a fluence of 60 $kJ \cdot m^{-2}$ the cells slowly disintegrated. Consistently, parallel assays in which we also measured the ability of the cells to divide and form colonies revealed cell deaths of 20% and 95% after light doses of 20 and 40 $kJ \cdot m^{-2}$, respectively.

Damage to plasma membrane of cells occurring over a shorter time period can be often assessed by loading cells with ^{51}Cr, and following release of label after a specific treatment. We therefore loaded endothelial cells with ^{51}Cr and ClAlPCS. Following a challenge with 82 $kJ \cdot m^{-2}$ of light we measured the kinetics of ^{51}Cr release. As shown in fig.2, ^{51}Cr was released slowly in the absence of light exposure, reaching 50% of total label by nearly 2 h. However, following the light exposure, release of ^{51}Cr was substantially more robust, reaching 50% in ~30 min. Therefore, photodynamic damage affecting nearly all the cells could be induced over a fairly short time.

The exposure of ClAlPCS-treated endothelial cells to light also released factors capable of inducing blood clotting. It was possible that simple destruction of the cells and concomitant release of

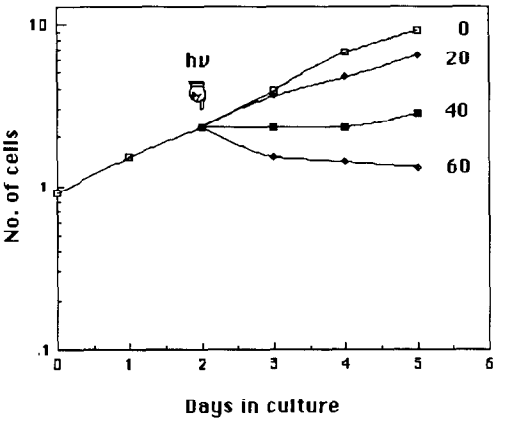

Fig.1. Photosensitized inhibition of endothelial cell growth. Bovine endothelial cells were plated at ~1×10^5 cells per 50 mm Petri dish. 1 day after plating, 4 μM ClAlPCS was added into the growth medium. 18 h later the medium was replaced with phosphate buffered saline (PBS) and the cells exposed to graded light fluences, as indicated (0–60 $kJ \cdot m^{-2}$). The growth medium was then readded and the cell number per dish determined at intervals by trypsinization and counting with a hemocytometer.

cellular contents could be solely responsible for this observation. Therefore, to test this possibility we performed parallel kinetic experiments measuring clotting factor release from ClAlPCS- and ^{51}Cr-loaded endothelial cells. As shown in fig.2, virtually no clotting factors were released in the dark. However, after light treatment, ~50% of the clotting factor activity was promptly released. In this case, the release reaction was virtually complete in less than 15 min. We concluded from these data that the photodynamic damage leading to release of ^{51}Cr occurred by a mechanism qualitatively different from that inducing clotting factor release.

The dose-response curves for ^{51}Cr release and clotting factor release at 4°C and 37°C also seem to bear out this conclusion. As shown in fig.3A and B, little photodynamic release of either ^{51}Cr or clotting factors was observed at 4°C. However, at 37°C substantial release of both substances occurred. Nonetheless, the energy needed to stimulate ^{51}Cr to 50% of maximum (~60 $kJ \cdot m^{-2}$) was much greater than the energy needed to stimulate clotting factor release to a similar extent (~20 $kJ \cdot m^{-2}$). Evidently, the release of ^{51}Cr is not as sensitive a criterion to cytotoxicity as is release of clotting factors. Thus, following a dose that kills

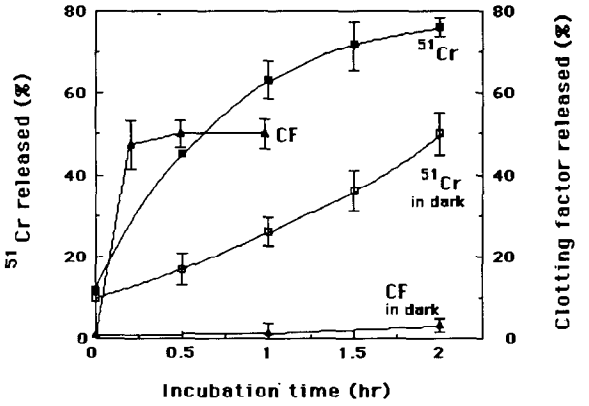

Fig.2. Kinetics of photosensitized-induced release of ^{51}Cr and clotting factors. Confluent cultures of endothelial cells in 50 mm Petri dishes were incubated for 18 h with 4 μM ClAlPCS and 1 μCi/ml ^{51}Cr. The cells were then rinsed with PBS and 2 ml (for ^{51}Cr) or 0.5 ml (for clotting factors), Hank's balanced salt solution (HBSS) containing glucose was then added. The plates were either exposed to 82 kJ·m^{-2} (filled symbols) or kept in dark (open symbols) at 23°C, and then incubated for various times at 37°C prior to assay of ^{51}Cr (□,■) or clotting factor release (△,▲) as described in section 2.

95% of the cells (40 kJ·m^{-2}) there was only 20% release of ^{51}Cr, while the release of clotting factors occurs in the biological dose range, saturating at ~50 kJ·m^{-2} (fig.3).

The mechanism of clotting factor release by endothelial cells is probably an active process (it does not occur at lower temperature), triggered by ClAlPCS-induced photodamage. In general, there are two pathways of protein secretion by eukaryotic cells: one is constitutive secretion and the other is regulated secretion [16]. Constitutive secretion occurs as soon as the protein is synthesized and transported to the cell surface. The regulated secretion involves the formation of a secretory vesicle that is released after an appropriate stimulus. These processes were shown to occur in cultured endothelial cells for von Willebrand's factor [17], which serves as a carrier for factor VIII:C in the plasma. In this case, regulated release was induced by thrombin or Ca^{2+} ionophore A23187. The induced release of von Willebrand's factor from the storage sites in endothelial cells is evoked within 10 min by various stimulators [18,19]. Photosensitization might interfere with the regulating mechanism of the clotting factors' secretion, resulting in massive release of these factors.

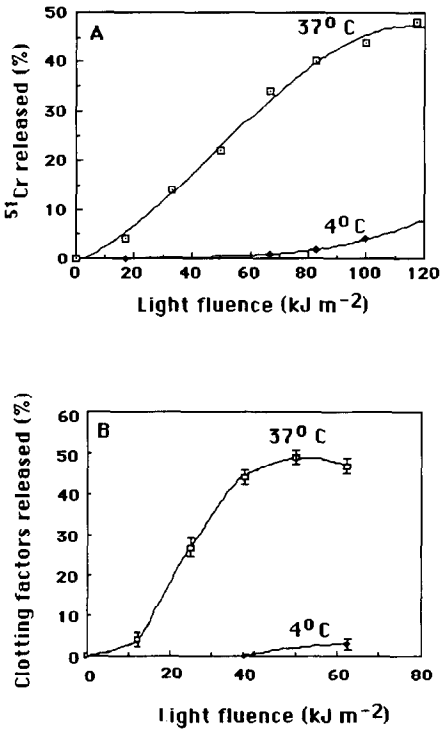

Fig.3. (A) Photosensitized-induced release of ^{51}Cr. For details see legend to fig.2. The plates were exposed to graded light fluences at room temperature and then incubated for 1 h at 37°C (□) or 4°C (♦). Triplicate dishes were used per data-point and the results are the mean value. SE were smaller than the symbols. Results are expressed as % activity released into medium after subtracting the value of unirradiated control (26%). (B) Photosensitized-induced release of clotting factors. Confluent cultures of endothelial cells in 50 mm Petri dishes were incubated with 4 μM ClAlPCS for 18 h. The cells were then rinsed with PBS and 0.5 ml HBSS was added. The plates were exposed to graded light fluences at room temperature (□) or 4°C (♦) and incubated for 10 min at 37°C (□) or 4°C (♦). The solutions from two replicate plates were pooled for clotting factor assay. Control, unirradiated dishes were used to determine spontaneous release, which was undetectable (see fig.2). To determine total activity, the cells were rinsed with PBS, freeze-dried at −20°C and then extracted with 0.5 ml HBSS for 1 h at 4°C. Results are expressed as percentage of total activity released into medium. Total activity was 8.5 ± 2.1% (using normal human plasma as 100%) in 1 × 10^6 cells and 3 × 10^6 cells were used per assay. Datapoints are the mean value ± SE of three separate experiments.

The present data clearly indicate that exposure of endothelial cells loaded with the photosensitizing drug ClAlPCS results in light-induced secretion of clotting factor activity by a mechanism quite distinct from simple cell destruction. The reasons

for this conclusion include the fact that the time course of light-induced clotting factor secretion is appreciably faster than release of ^{51}Cr, and the fact that the energy profiles of both processes are different. It is clear that at least two processes directly controlled by endothelial cells may lead to local blood clotting. One of these is the release of clotting factors, possibly including factor VIII:C. The second process is release of tissue factor by cell destruction. It is of course true that merely releasing factor VIII:C would not necessarily generate a local clot. However, activation of factor VIII:C in association with other processes cannot be excluded. Finally, tissue factor by itself, acting on factor X, would directly cause clotting. The contributions of the different processes can presumably be distinguished by the differential time courses of clotting factor secretion and ^{51}Cr secretion.

We conclude that the basis for effective photodynamic therapy of tumors may indeed involve direct action on the level of endothelial cells lining the vessels and forming the capillaries in susceptible tumors. This is a novel finding, which we anticipate will prove important for devising more effective therapeutic protocols.

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REFERENCES

[1] Dougherty, T.J. (1984) CRC Crit. Rev. Oncol. Hematol. 2, 83–116.
[2] Wilson, C.B. and Jeeves, W.P. (1987) Photomedicine (Ben-Hur, E. and Rosenthal, I. eds) vol.2, pp.127–177, CRC Press, Boca Raton, FL.
[3] Selman, S.H., Kreimer-Birnbaum, M., Klaunig, J.E., Goldblatt, P.J., Keck, R.W. and Britton, S.L. (1984) Cancer Res. 44, 1924–1927.
[4] Selman, S.H., Kreimer-Birnbaum, M., Goldblatt, P.J., Anderson, T.S., Keck, R.W. and Britton, S.L. (1985) Cancer Res. 45, 6425–6427.
[5] Star, W.M., Marjnissen, H.P.A., Van den Berg-Blok, A.E., Versteeg, J.A.C., Franken, K.A.P. and Reinhold, H.S. (1986) Cancer Res. 46, 2532–2540.
[6] Henderson, B.W., Waldow, S.M., Mang, T.S., Potter, W.R., Malone, P.B. and Dougherty, T.J. (1985) Cancer Res. 45, 572–576.
[7] Nelson, J.S., Liaw, L.H. and Berns, M.W. (1987) Photochem. Photobiol. 46, 829–835.
[8] Berenbaum, M.C., Hall, G.W. and Hayes, A.D. (1987) Br. J. Cancer 53, 81–89.
[9] Banerjee, D.K., Orenberg, R.L., Youdim, M.B.H., Heldman, E. and Pollard, H.B. (1985) Proc. Natl. Acad. Sci. USA 82, 4702–4706.
[10] Pollard, H.B., Lelkes, P.I., Orenberg, R., Heldman, E., Banergee, D. and Yudin, M. (1988) Science, in press.
[11] Ben-Hur, E. and Rosenthal, I. (1985) Int. J. Radiat. Biol. 47, 145–147.
[12] Ben-Hur, E., Rosenthal, I., Bown, S.G. and Phillips, D. (1987) Photomedicine (Ben-Hur, E. and Rosenthal, I. eds) vol.3, pp.1–17, CRC Press, Boca Raton, FL.
[13] Ben-Hur, E. (1987) Photobiochem. Photobiophys. suppl. 407–420.
[14] Selman, S.H., Kreimer-Birnbaum, M., Chaudhuri, K., Garbo, G.M., Seaman, D.A., Keck, R.W., Ben-Hur, E. and Rosenthal, I. (1986) J. Urol. 136, 141–145.
[15] Thompson, A.R. and Harker, L.A. (1983) Manual of Hemostasis and Thrombosis, 3rd edn, Davis Company, Philadelphia, PA.
[16] Kelly, R.B. (1985) Science 230, 25–32.
[17] Sporn, L.A., Marder, V.J. and Wagner, D.D. (1986) Cell 46, 185–190.
[18] Levine, J.D., Harlan, J.M., Harker, L.A., Josheph, M. and Counts, R.B. (1982) Blood 60, 531–534.
[19] Loesberg, C., Gonsalves, M.D., Zanbergen, J., Willems, Ch., Van Aken, W.G., Stel, H.V., Van Mourik, J.A. and De Groot, P.G. (1983) Biochim. Biophys. Acta 763, 160–168.