Response of rat incisor mesenchymal cells to doxorubicin after bleomycin pretreatment

Jon Einar Dahl
Department of Pathology, Faculty of Dentistry,
University of Oslo, Oslo, Norway

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Bleomycin is a possible synchronizing agent of dividing cell populations in vivo. Utilizing this property, we studied doxorubicin-induced alterations in incisor mesenchymal cells by light microscopy in rats pretreated with bleomycin. Two experimental groups were given 25 mg/kg and 75 mg/kg bleomycin, respectively, followed by a dose of 5 mg/kg doxorubicin after 32 h. At that time, the synchronized cells were theoretically entering the synthesis phase of the cell cycle, in which they are most vulnerable to doxorubicin. Animals given 75 mg/kg bleomycin had indications of cell synchronization, resulting in increased destruction of the basal pulp 1 day after doxorubicin administration. Two odontoblast/dentinal lesions were seen on the 5th day of observation, each induced by injury to cells that were late preodontoblasts at the time of bleomycin and doxorubicin injection. The histomorphological alteration seen after the lowest bleomycin dose in combination with doxorubicin included necrosis of basal pulp cells after 1 day and dentinal dearrangements after 5 days, corresponding to what has previously been found in animals given doxorubicin only. □ *Antineoplastic agents; cell cycle; dental pulp; dentinogenesis; drug combination*

Jon Einar Dahl, Department of Toxicology, National Institute of Public Health, Geitmyrsveien 75, N-0462 Oslo 4, Norway

The anti-tumor antibiotic bleomycin (1) has been found reversibly to arrest cycling cells in the G_2 (postsynthetic-premitotic) phase in vitro (2, 3), leading to an accumulation of cells in this phase of the cell cycle. Discontinuation of the bleomycin effect enabled the temporarily blocked cells to re-enter the cell cycle and divide simultaneously. Bleomycin is thus suggested to have a synchronizing effect in vivo on human tumor cells (4) and rat incisor basal pulp cells (5). If administered in the vulnerable phase, treatment with other phase-specific cytotoxic agents will have more pronounced effects in a synchronized than in a normal cell population (4). The influence of a single dose of the anti-tumor agent doxorubicin on the rat incisor mesenchymal cells has previously been studied (6). Necrosis of proliferating cells was found after 1 day, followed by a dose-dependent diminished circumpulpal dentin production and deposition of irregular dentin in the pulp, as observed after 5 days (6). By intercalating into nuclear DNA and blocking of the transcription (7, 8), doxorubicin inhibits DNA and RNA synthesis. Further, doxorubicin is reported to cause single-strand breaks and fragmentation of DNA, most probably mediated by a free radical mechanism (9). Cells in their synthesis phase are most vulnerable to the drug, although doxorubicin is not regarded as a strictly phase-specific drug (10).

The objectives of the present in vivo study were to synchronize rat incisor mesenchymal cells by bleomycin pretreatment and to investigate the effects of a single dose of doxorubicin administered during the estimated synthesis phase of the synchronized cells.

Materials and methods

Thirty-eight female rats (Wistar, Møllegaards Breeding Centre Ltd, Skensved, Denmark) with a mean weight of 198 g were used in the experiments. The animals were

grouped, dosed, and killed as outlined in Table 1. Bleomycin (Bleomycin Lundbeck, H. Lundbeck & Co A.S., Copenhagen, Denmark) and doxorubicin hydrochloride (Adriamycin®, Farmitalia, Montedison Farmaceutica, Italy) were dissolved in isotonic saline and sterile water, respectively. All injections were given in the tail vein under light ether anesthesia, and the volumes given were 0.5 ml/200 g (injection 1) and 0.25 ml/200 g (injection 2). The animals killed after 24 h were killed by perfusion. Half of the rats given two injections were decapitated under ether anesthesia.

The maxillae were freed from soft tissue, divided by a median line incision and, after removal of the lateral nasal wall, fixed in 2% glutaraldehyde in phosphate buffer for 3 days. The remaining animals were killed by perfusion fixation. Under ether-ethanol anesthesia the thoracic cavity was opened and a cannula inserted in the ascending aorta through an incision of the left ventricle. Outflow was established through an incision of the right atrium. Prerinse was performed with physiologic saline solution for 2 min followed by perfusion with 2% glutaraldehyde in phosphate buffer for 10 min. The maxillae were removed and immersed in glutaraldehyde for 1 day. All maxillae were demineralized in an aqueous solution prepared from equal amounts of 44% formic acid and 20% sodium citrate. The incisors were removed with surrounding bone, sectioned transversally into 3-mm-long apico-incisal pieces, postfixed in 1% osmium tetroxide for 2 h, and embedded in Vestopal W (Chemische Werke Huls AG, Mari, FRG) (11). Sixty to 80 longitudinal sections 1–2 μm thick were taken from each specimen and stained with methylene blue–azure II–basic fuchsin (12). The sections were investigated by light microscopy.

Results

In control animals the mesenchymal cells of the incisors were grouped as proliferative cells (basal pulp cells, early and late preodontoblasts) and non-proliferative cells (pulp cells, young odontoblasts, and odontoblasts), as previously described (5, 13) (Fig. 1a). In animals given 25 mg/kg bleomycin, the morphology of basal pulp cells and preodontoblasts was similar to that of the controls after 24 h. A few mitotic figures were observed in both cell populations. An increased number of cells in mitosis (Fig. 2) and some necrotic cells were observed in the basal pulp of rats injected with 75 mg/kg bleomycin. Young odontoblasts and odontoblasts of rats in both groups seemed to be unaffected by the drug.

An evaluation of the experimental animals 1 day after injection of doxorubicin showed morphological alterations of the proliferative (apical) part of the incisors (Fig. 1b). In rats from both groups I and II areas of liquefaction necrosis, pyknotic nuclei, and many punched-out areas containing fragmented nuclei were observed in both immersion- and perfusion-fixed progenitive pulp tissue (Fig. 3). The alterations were most severe in the region adjacent to the preodontoblasts of group I and more widespread in the group II basal pulp. A few necrotic preodontoblasts were found in rats of group I, whereas in group II animals most early preodontoblasts were lost. Young odontoblasts and odontoblasts of group I rats appeared like those of control animals. A small region of shortened

Table 1. Presentation of the experimental procedure

	Time point	Group I	Group II	Control
Total no. of animals	0	14	14	10
Injection 1	0	Bleomycin, 25 mg/kg	Bleomycin, 75 mg/kg	Isotonic saline
No. of animals killed	24 h	2	2	2
Injection 2	32 h	Doxorubicin, 5 mg/kg	Doxorubicin, 5mg/kg	Isotonic saline
No. of animals killed	32 h + 1 day	6	6	4
No. of animals killed	32 h + 5 days	6	6	4

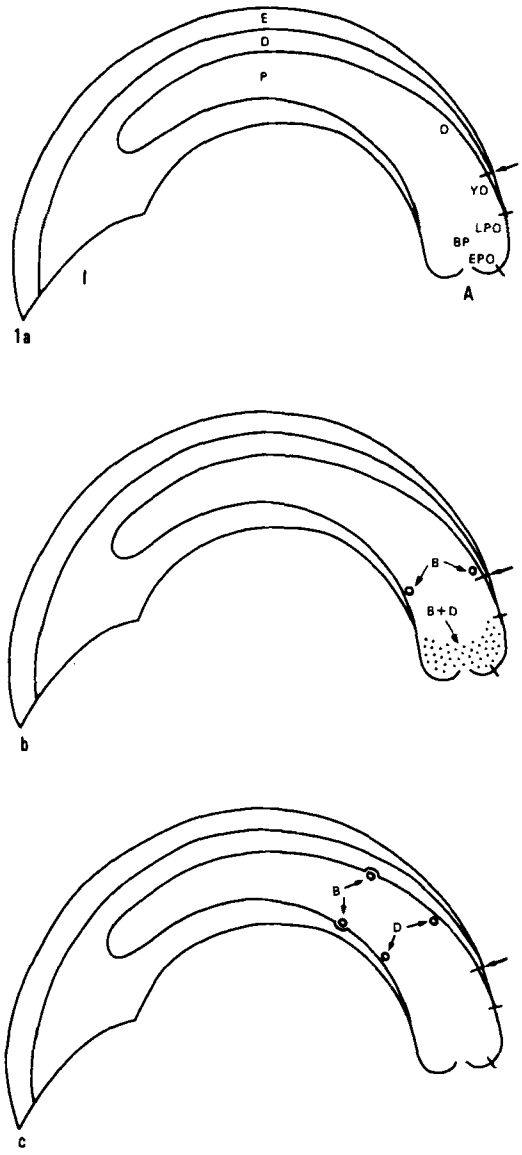

Fig. 1. Schematic presentation of longitudinal sections of maxillary incisors from control rat (1a) and experimental rats of group II 1 day (1b) and 5 days (1c) after doxorubicin injection. *1a.* A labial layer of enamel (E) covers the dentin (D), which surrounds the pulp. The mesenchymal cells comprise basal pulp (BP) and pulp cells (P), early and late preodontoblasts (EPO, LPO), young odontoblasts (YO), and odontoblasts (O). I = incisally; A = apically. Arrow = startpoint of enamel production. *1b.* The locations of doxorubicin effects after bleomycin pretreatment (B + D signed dots) and the bleomycin-induced odontoblast/dentinal lesion (B) are shown. *1c.* Presentation of odontoblast/dentinal lesions induced by bleomycin (B) and doxorubicin (D) injections.

odontoblasts, some of which had a depolarized nucleus, and a pulpal island of predentin were noted just incisally to the young odontoblast/odontoblast borderline in group II (Figs. 1b and 4). Some cytoplasmic vacuolization was observed in the odontoblasts of immersion-fixed animals.

On the 5th day of observation, the morphology of proliferative pulp cells and preodontoblasts of all experimental animals concurred with that of the controls. A zone of shortened and wavy odontoblasts with displaced nucleus pulpally lined by irregular predentin was seen just incisally to the young odontoblasts in both experimental groups. A few cells were seen entrapped in the pulpal predentin (Figs. 1c and 5). In addition, a zone of similar odontoblast dearrangment with pulpal predentin was found approximately 2000 μm more incisally in the group II animals (Fig. 1c).

Discussion

Perfusion fixation was performed in half the animals. This procedure gave a better fixation quality, especially of the cells surrounded by dentin, compared with immersion fixation (5). It also eliminated the cytoplasmic vacuoles, which have been interpreted as a fixation artifact (5, 6).

An increased number of cells in mitosis, as seen 24 h after bleomycin treatment in group II animals (75 mg/kg), is indicative of synchronization of the cell population (5). The time interval between mitosis and synthesis phase of the cell cycle (for example, the G_1 phase) is estimated to be 8 h 40 min and 7 h 30 min for preodontoblasts and basal pulp cells, respectively (13). Theoretically, the preodontoblasts and basal pulp cells were consequently entering the synthesis phase when doxorubicin was administered 8 h after the increased number of mitotic figures was observed (equals 32 h after bleomycin injection) and should thus be must vulnerable to doxorubicin (10).

The greater spread of necrosis within the basal pulp of group II animals compared with that found after a single dose of 5 mg/kg doxorubicin (6) must be interpreted as a

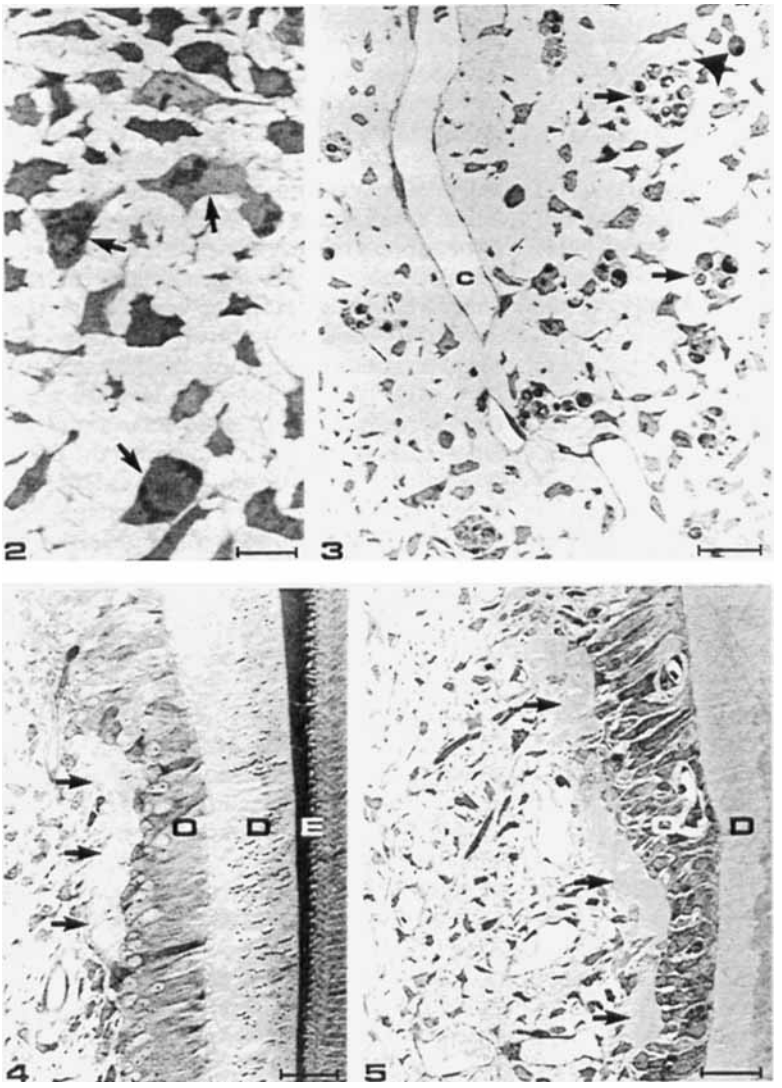

Fig. 2. Photomicrograph of section from the basal pulp of rat given 75 mg/kg bleomycin and killed after 24 h, showing accumulation of cells in mitosis (arrows). Vestopal W-embedded. Bar, 10 μm.

Fig. 3. Photomicrograph of section from the basal pulp of rat given 75 mg/kg bleomycin followed by 5 mg/kg doxorubicin (group II) 1 day after doxorubicin injection. Pyknotic nucleus (arrow head) and punched-out areas of necrotic cells (arrow) are observed. C = capillary. Bar, 17 μm.

Fig. 4. Photomicrograph of odontoblast (O) region of section taken from the same (group II) animal as in Fig. 3 shows the bleomycin-induced lesion with shortened odontoblasts and a pulpal island of predentin (arrows). D = predentin and dentin; E = enamel. Bar, 25 μm.

Fig. 5. Photomicrograph of section of group II rat 5 days after doxorubicin injection shows the doxorubicin-induced lesion with pulpal predentin (arrows) and altered odontoblasts (O). D = predentin and dentin. Bar, 25 μm.

manifestation of increased sensitivity of the cell population after the bleomycin administration. Synchronization of the cell division, leading to an increased number of cells in the S-phase at the time of doxorubicin injection is a likely explantion. However, some cytotoxic potentiation caused by bleomycin–doxorubicin interaction cannot be totally disregarded, since a slight synergistic effect was noted when cells in vitro were exposed to the two drugs simultaneously (14). The existence of a time interval between the two drug

administrations is, however, a point in favor of the former explanation. The cytotoxic effect observed in the basal pulp after 1 day in group I animals (25 mg bleomycin/kg) was equal to that seen in animals given a single injection of doxorubicin only (6). This was not an unexpected observation, since this bleomycin dose seemed to have no histomorphologic effect on the basal pulp, as noted previously (5).

The number of preodontoblasts in mitosis increased 24 h after the injection of bleomycin, which indicated that synchronization was achieved. However, the preodontoblast region was necrotic in both bleomycin-pretreated and not pretreated animals (6), and, likewise, scattered necrotic late preodontoblasts were observed in both regimens. It was therefore difficult to interpret the immediate reaction within the preodontoblast zone, but preodontoblasts have been found to be less reliable than basal pulp in grading cytotoxic effects (15), owing to the considerably smaller number of cells.

Five days after doxorubicin administration, two odontoblast/dentinal lesions were present in the group II animals. The size and location of the most apical one of these were similar to those of the solitary lesion observed in the group I and after single injection of doxorubicin (6). A comparable lesion was formed in cyclophosphamide-injected rats and believed to be caused by injury to cells that were late preodontoblasts at the time of injection (16), which also resulted in diminished circumpulpal dentin deposition (17). In animals given 20 mg/kg doxorubicin, the odontoblasts of the lesion were young odontoblasts and late preodontoblasts at the time of drug injection (18). During the time needed for accumulation of doxorubicin in the cells, late preodontoblasts differentiated into non-proliferating young odontoblasts, which were more resistant to doxorubicin (18, 19), leading to this non-lethal cell injury. As expected, the non-lethal effect of doxorubicin was not increased by bleomycin pretreatment, since this effect was related to non-proliferating cells. The most incisally positioned lesion of the group II animals must have been caused by bleomycin, since only one lesion was formed in the incisor of animals given a single dose of doxorubicin (6). The dearrangement of young odontoblasts observed 56 h after bleomycin injection was a likely precursor to the lesion. The odontoblasts within the lesions appeared shortened with depolarized nucleus, enabling the cells to secrete predentin into the pulp (20), which has been regarded a reparative event induced by nearby cell necrosis (16).

The progenitive pulp cells and the preodontoblasts in this study had regenerated after 5 days, as was also observed after a single dose of doxorubicin (6). However, the basal pulp necrosis was more extensive in the present study. The effects of the combination of bleomycin and doxorubicin on the proliferative cells must therefore be regarded as transitory.

To conclude, sequential administration of 75 mg/kg bleomycin and doxorubicin resulted in necrosis, which was especially intensified within the basal pulp compared with animals receiving a single dose of doxorubicin. An increased sensitivity to doxorubicin in a bleomycin-synchronized cell population seems to be the most likely explanation. In addition, each drug administration resulted in separate odontoblast/dentinal lesion due to injury of late preodontoblasts.

References

1. Umezawa H, Maeda K, Takeuchi T, Okami Y. New antibiotics, bleomycin A and B. J Antibiotics Ser A 1966;19:200–9.
2. Barranco SC, Humphrey RM. The effects of bleomycin on survival and cell progression in Chinese hamster cells in vitro. Cancer Res 1971;31:1218–23.
3. Nagatsu M, Richart RM, Lambert A. Effects of bleomycin on the cell cycle of Ehrlich ascites carcinoma. Cancer Res 1972;32:1966–70.
4. Barranco SC, Luce JK, Romsdahl MM, Humphrey RM. Bleomycin as a possible synchronizing agent for human tumor cells *in vivo*. Cancer Res 1973;33:882–7.
5. Dahl JE, Kjærheim Å. Evaluation of rat incisor mesenchymal cells in bleomycin injected animals fixed by immersion or perfusion. Scand J Dent Res 1985;93(6) (in press).

6. Dahl JE. Influence of doxorubicin on rat incisor mesenchymal cells. Scand J Dent Res 1984;92:6–13.
7. Meriwether WD, Bachur NR. Inhibition of DNA and RNA metabolism by daunorubicin and Adriamycin in L 1210 mouse leukemia. Cancer Res 1972;32:1137–42.
8. di Marco A. Adriamycin (NSC-123127): Mode and mechanism of action. Cancer Chemother Rep 1975;6(Part 3):91–106.
9. Aubel-Sadron G, Londos-Gagliardi D. Daunorubicin and doxorubicin, anthracycline antibiotics, a physiochemical and biological review. Biochimie 1984;66:333–52.
10. Bhuyan BK, Blowers CL, Shugars KD. Lethality of nogalamycin, nogalamycin analogs, and adriamycin to cells of different cell cycle phases. Cancer Res 1980;40:3437–42.
11. Ryter A, Kellenberger E. L'inclusion au polyester pour l'ultramicrotomie. J Ultrastruct Res 1958; 2:200–14.
12. Humphrey CD, Pittman FE. A simple methylene blue-azure II-basic fuchsin stain for epoxy-embedded tissue sections. Stain Technol 1974;49: 9–14.
13. Dahl JE. Proliferation and migration of rat incisor mesenchymal cells. Scand J Dent Res 1983;91:335–40.
14. Drewinko B, Loo TL, Brown B, Gottlieb JA, Freireich EJ. Combination chemotherapy *in vitro* with adriamycin. Observation of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymfoma cells. Cancer Biochem Biophys 1976;1:187–95.
15. Dahl JE. Immediate and delayed effects of repeated doxorubicin injections on rat incisor mesenchymal cells. Acta Odontol Scand 1985;43:155–62.
16. Koppang HS. Histomorphologic investigations on the effect of cyclophosphamide on dentinogenesis of the rat incisor. Scand J Dent Res 1973;81:383–96.
17. Koppang HS. Effect of cyclophosphamide on dentinogenesis of the rat incisor: fluorescence microscopic and microradiographic investigations. Scand J Dent Res 1981;89:59–70.
18. Dahl JE, Koppang HS. Renewal and migration of rat incisor mesenchymal cells after doxorubicin administration. Acta Odontol Scand 1985;43:97–102.
19. Bhuyan BK, Fraser TJ, Day KJ. Cell proliferation kinetics and drug sensitivity of exponential and stationary population of cultured L1210 cells. Cancer Res 1977;37:1057–63.
20. Pliess G. Wirkungen der Cytostatica auf die Zahnentwicklung der Albinoratte. III. Mitteilung. Morphologische Veränderungen an den Insisoren der erwachsenen Ratte nach Aminopterin, Colcemid, E39 und B518 Asta. Frankf Z Pathol 1958;69:437–51.

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