Scanning Microscopy

Volume 4 | Number 1 Article 14

11-20-1989

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Scanning Microscopy, Vol. 4, No. 1, 1990 (Pages 143-149) 0891-7035/90$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

CHANGES IN THE ESOPHAGEAL EPITHELIUM IN RABBITS TREATED BY CIS-DICHLORODIAMMINEPLATINUM AS STUDIED BY ELECTRON MICROSCOPY

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(Received for publication August 20, 1989, and in revised form November 20, 1989)

Abstract

The esophageal mucosa of the rabbit was investigated after a single dose of 5 mg Cis-Dichlorodiammineplatinum (Cis-DDP). Specimens were taken for scanning electron microscopy, transmission electron microscopy, and light microscopy. Examination was performed daily for 20 consecutive days. A cytotoxic effect was observed already the first day after injection with an intracellular oedema. Thereafter the height of the esophageal epithelium and the basal cell layer steadily decreased to a minimum day 11. This parallels the damaged microridges and an increased cell loss as revealed by scanning electron microscopy. At the end of the observation period the esophageal mucosa had completely restituted.

Key Words: Esophagus, mucosal epithelium, Cis-dichlorodiammineplatinum (Cis-DDP), light microscopy, scanning electron microscopy, transmission electron microscopy.

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Introduction

Squamous cell carcinoma of the esophagus is a disease with poor prognosis. With the ordinary treatment modalities, radiotherapy or surgery, 5 year survival is below 5%. Due to this fact other treatment modalities have been tested. Cytostatic drugs have been added to the treatment before the operation, e.g., Mitomycin and Bleomycin. In the last few years Cisplatin® (Cis-dichlorodiammineplatinum, Cis-DDP) has been added to the therapeutic arsenal. Cis-DDP was the first member of a new class of potent anticancer drugs, which showed high activity against squamous cell carcinoma in the head and neck region and in the esophagus (Kish et al. 1984, Decker et al. 1983). The drug was used in combination with ionizing radiation for esophageal carcinoma and this mode of action improved treatment results convincingly. Then the question arose as to why the drug was so successful in destroying cancer cells, with minor effects upon the normal tissues of the body, since some normal tissues exhibit a significantly higher uptake than tumor cells, e.g., the liver and skin, which are known to be major depots for the platinum drug (Wolf and Manaka 1977). Also the drug had no negative influence on the hepatic function or skin cells. In the radiotherapy treatment, even optimized, the acute effects of the normal tissues are the limiting factors. Moreover, the absence of data regarding the toxicity of concomitant treatment with radiation and Cis-DDP, is conspicuous. Therefore, earlier research with ultrastructural methods has been performed with Cis-DDP and fractionated irradiation on the esophageal mucosa in rabbits (Albertsson et al. 1987a, b). A damage effect was observed with edema, increased cell loss, and loss of microridges. The effect of the drug alone was observed within the lower part of the esophagus where no absorbed dose of radiation could be measured. However, supplementary examinations with only Cis-DDP given to the animals had to be performed. Thus, any possible influence from ionizing radiation or anaesthesia is excluded. This paper is part of a research program concerning the effects of Cis-DDP and radiation on different tissues. The aim of this study was to investigate the toxicity of a single dose Cis-DDP on the esophageal mucosa.

Materials and methods

Animals

Thirtyfour full-grown rabbits weighing between 1.8 - 2.3 kg were selected for this study. Fourteen

animals acted as untreated controls.

Drug

Cis-dichlorodiammineplatinum (II), (Cis-DDP, Cis-platinum), (Platinol, Bristol Myers Company) was dissolved in normal saline at a concentration of 0.5 mg/ml.

Experiments

The animals received 5 mg Cis-DDP intraperitoneally. One rabbit each day was examined from day one to day twenty after the injection. The animals were sacrificed by a blow on the skull in order to avoid pharmacological side-effects. The esophagus was dissected out in its entire length (7-9 cm). Samples for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were taken from two sites: the upper and lower part respectively.

Preparation for SEM

The specimens for SEM examination were not rinsed. They were fixed in 2.5% glutaraldehyde (in 0.15 M cacodylate buffer, pH of the solution = 7.3) for 12 hours. They were then transferred into the same buffer, and were later osmium-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for two hours. After dehydration with a graded series of ethanol, the preparations were transferred to Freon TF 618. The specimens were later critical point dried in a Balzer-000 critical point dryer. They were then sputter-coated with gold, plus palladium, in a Polaron coating unit (E5000) and examined in a Philips 515 SEM operating at 20 kV.

Preparation for TEM

The samples were fixed, treated with osmium tetroxide, and dehydrated in ethanol in the same manner as for the SEM preparations. The samples were then embedded in Vestopal W or Epon. Ultrathin sections were cut out and contrasted with lead citrate and uranyl acetate. For TEM examination, a JEOL 2000X electron microscope was used both within the low and high range of magnification.

Scoring system

The score for loosened microridges was based on the number as calculated from SEM pictures (at 5000X) in an area of 17 x 11 cm; score 0 = 0-50, score 1: approximately 100, score 2: approximately 200, score 3: greater than 250. The score for cell loss was based on the number of loosening cell flakes in an area of 17 x 11 cm on a SEM micrograph (at 100X); score 0: less than 50; score 1 = 51-100; score 2 = 101-150; score 3 greater that 150.

Measurement of epithelial thickness

The thickness measurements were performed by a Leitz 12.5x measurement ocular (1 mm divided into 100 parts). From each animal 8-10 sections were investigated. Due to undulations of the epithelium 20 measurements at various sites from each animal were made.

Results

Reference animals

Figs. 1 and 2 present the TEM and SEM micrographs for the normal mucosa. The thickness of the squamous epithelium outlining the esophagus in this control material is 126 ± 20 micrometers (Fig. 3). According to Rhodin (1963), it consists of three strata: a basal, an intermediate, and a superficial stratum. In the basal stratum around 10 cell layers can be identified (as shown later in a TEM micrograph, Fig. 9). According to Leblond et al. (1964), only those cells in connection with basal lamina are capable of proliferation. In that sense, only a single basal stratum can be identified. However, a basal stratum exist which is defined according to the ultrastructural appearance (Rhodin 1963). Even though separate cells with large nuclei are observed in the intermediate stratum, it is possible to identify a basal stratum which can be measured as cell layers and height . The basal stratum is then defined as those cells which are columnar with a central nucleus, normally round, occupying the major part of the cell. The nuclei usually have a prominent nucleolus and a thin peripheral layer of chromatin. Rich amounts of mitochondria and ribosomes are situated in the cytoplasm. The cells are connected to each other by desmosomes and to the basal lamina with hemidesmosomes (Fig. 1).

Fig. 1. TEM micrograph of normal esophageal mucosa. Hemidesmosomes (arrows).

Fig. 2 a-c. SEM micrographs of normal esophageal mucosa showing cell loss (arrow) in **a**, microridges in **b**, and gland opening in **c**. Numerous bacteria can be observed on the mucosal surface.

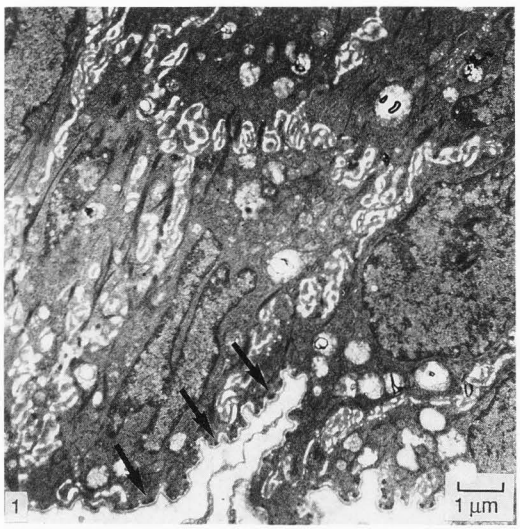
In the intermediate stratum (10-20 cell layers), the cells gradually flatten. Compared to the basal stratum, fewer mitochondria and ribosomes are seen in the cytoplasm. In the apical part (10-20 cell layers), the majority of cells have lost their nuclei and the cytoplasm lacks cell organelles. Towards the lumen, the intercellular space gradually closes up. SEM of the normal mucosal surface shows polygonal cells regularly arranged and the cell borders are easily identified (Figs. 2a, b, c). Microridges cover the surface in a pattern which varies from one cell to another. Under normal physiological conditions, cell loss from the surface occurs constantly. The cells often loosen in flakes composed of a complete group. Bacteria are frequently seen on the surface, mainly rods, in a moderate amount.

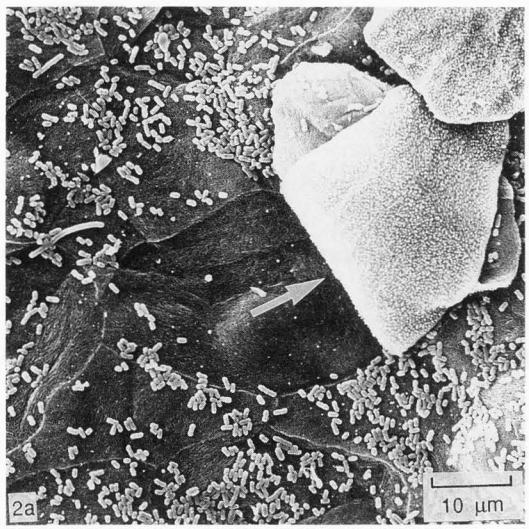
Cis-DDP treated animals

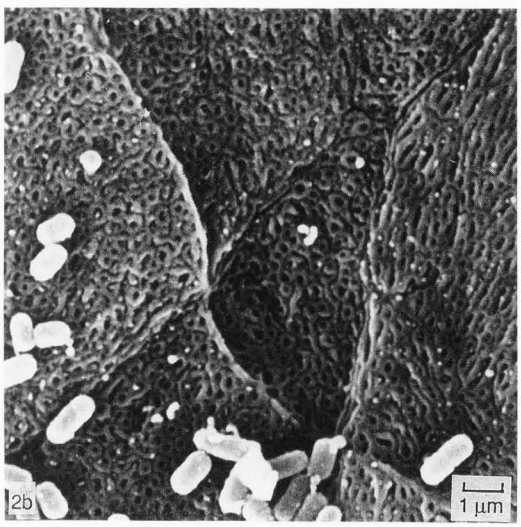
The thickness of the mucosal epithelium was calculated from light microscopy (LM) observations (Fig. 3). Although we are well aware of the difficulties in giving a statistically correct evaluation when only one animal per day has been examined, our aim in this study was to illustrate the course of events over a period of time. The results are presented in Fig. 3. One day after injection, the thickness of the mucosal epithelium had increased from 126 to 136 µm. This increase was sustained for two days (136 µm on day 1, 132 µm on day 2). Thereafter, the thickness gradually decreased and reached a minimum around day 10 after drug injection (79 µm). Towards the end of the observation period, the mucosal epithelium had regained its normal height. The number of cell layers was calculated on TEM and the course of events during the 20 days after drug injection is presented in Fig. 4. There seems to be a tendency towards a dip around days 6-10 and during the subsequent ten days a gradual increase is seen.

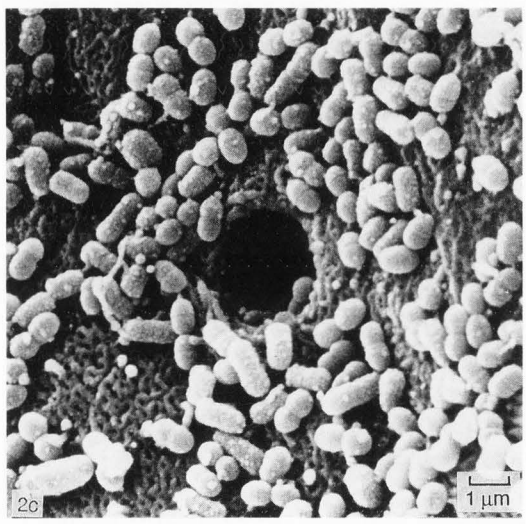
SEM

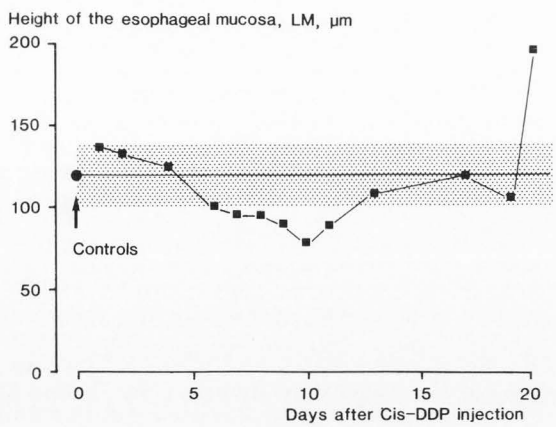
The extent of cell loss was scored on a specimen taken each day after drug injection for 20 days,











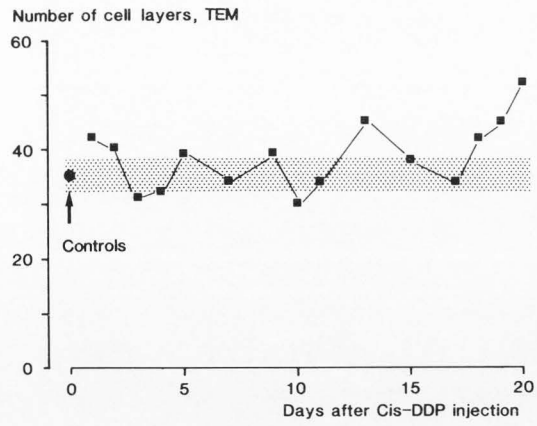
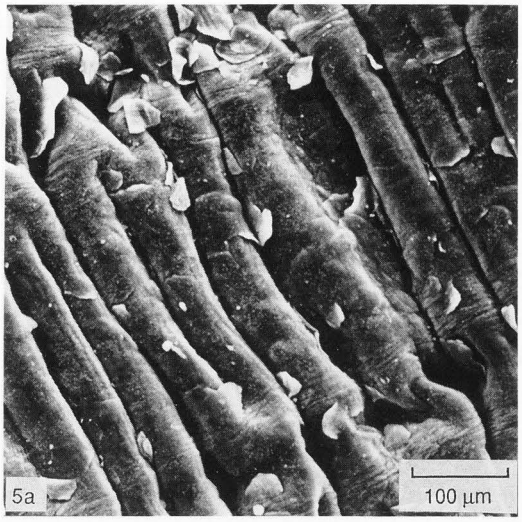
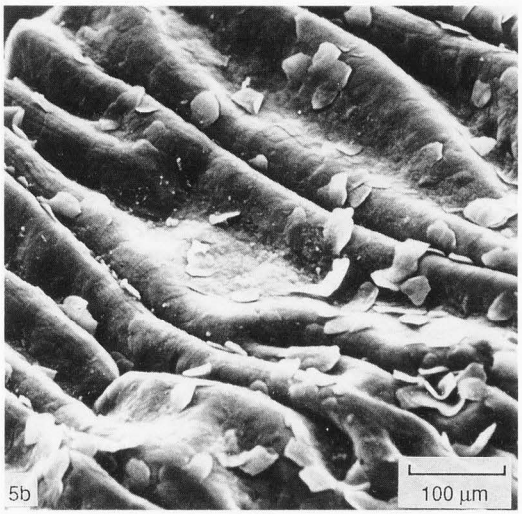
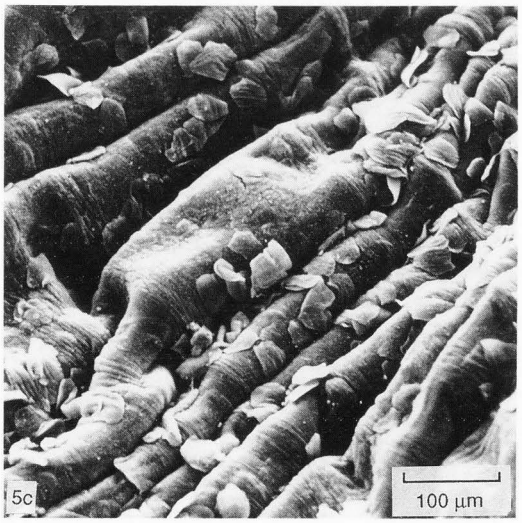


Fig. 3 (at left). Height of esophageal mucosa as calculated from LM micrographs. Dotted area represents standard deviation of the control values.
Fig. 4 (at right). Number of cell layers as calculated from TEM micrographs.







Figs. 5 a-c. SEM micrographs illustrating cell loss. **a, b, and c** are for scores 1, 2, and 3 respectively.

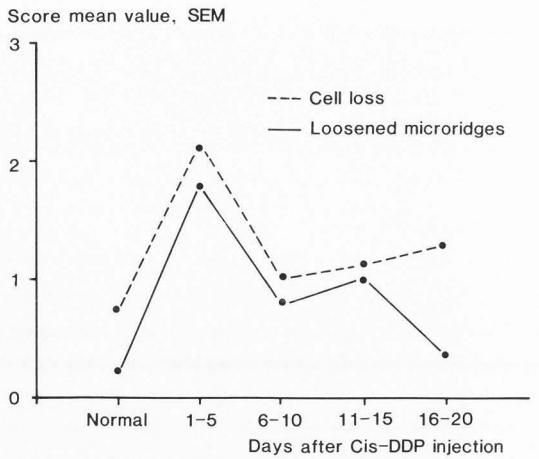


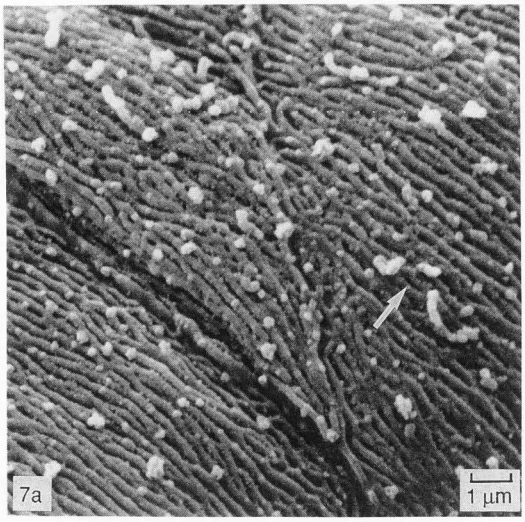
Fig. 6. Score curve for cell loss and loosened microridges.

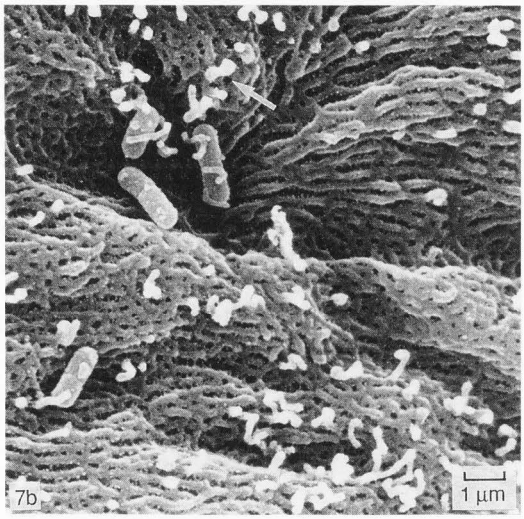
according to the criteria listed in Material and Methods and the results are presented in Figs. 5a-c and Fig. 6. Since scoring of the surface structure is a rough estimation which necessarily gives high amplitudes in evaluation, the mean value for four different time periods is presented. The scoring system is the same as presented earlier (Albertsson et al. 1987a). For four untreated rabbits, the mean value for cell loss does not reach 1, which may seem strange. However, in order to achieve uniformity, the same score as published earlier is maintained. Since the score only affects the results in a relative way, the final outcome does not change. During the first five days, the cell loss was increased to a score mean value of 2.1. Thereafter, the cell loss was about the same as in the normal physiological condition (1.0).

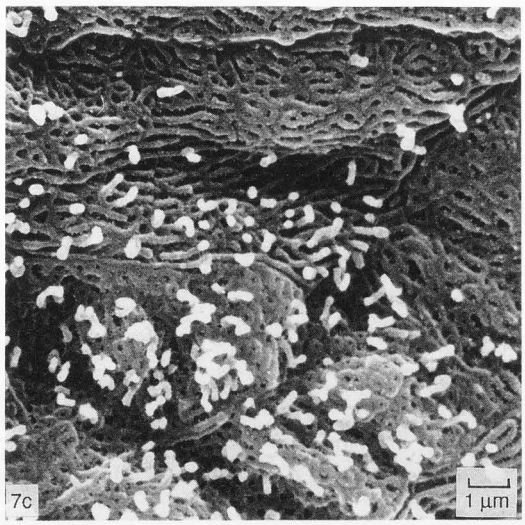
On the surface of the mucosa, microridges are arranged in a pattern that varies from one cell to another and even within the same cell (Fig. 2). Occasionally microridges loosen at one end and take the form of knobs or small snakes (Fig 7a-c). This was previously described after treatment with Cis-DDP and fractionated irradiation (Albertsson et al. 1987a, b). Scoring was performed which illustrated that in this series, the damaged microridges were more pronounced during the days 1-5 with a score mean value of 1.8 (Fig 6). Already on days 6-15, the number was reduced and for the last few days of the observation period, the score mean value of damaged microridges was about the same as in the control material. An increase in the cell loss and loosened microridges is interpreted as an acceleration of the normal physiological course of events, and it seems reasonable that the two are related. The discharge of cells from the surface is a normal phenomenon and a part of the physiological steady state in the mucosa where the basal cells multiply, differentiating on the way to the luminal layers where they are sloughed off. The average migration time in rats is about 9-11 days (Bertalanffy 1960). Both the thickness of the mucosal epithelium (Fig. 3) and the score curve for cell loss (Fig. 6) indicate that this is the same for rabbits.

TEM

The height of the esophageal mucosa and the amount of basal cell layers were calculated from the







Figs. 7 a-c. SEM micrographs illustrating loosening microridges. **a** = score 1; **b** = score 2; **c** = score 3.

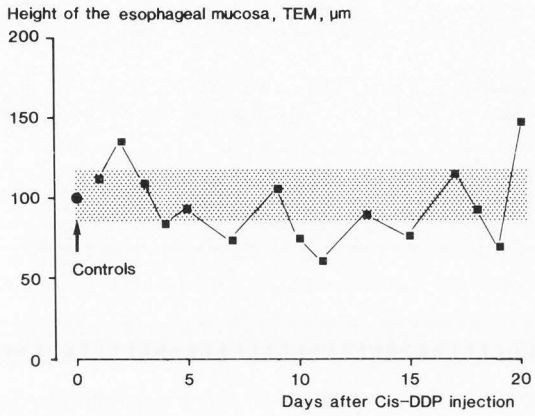


Fig. 8. Height of esophageal mucosa as calculated from TEM micrographs.

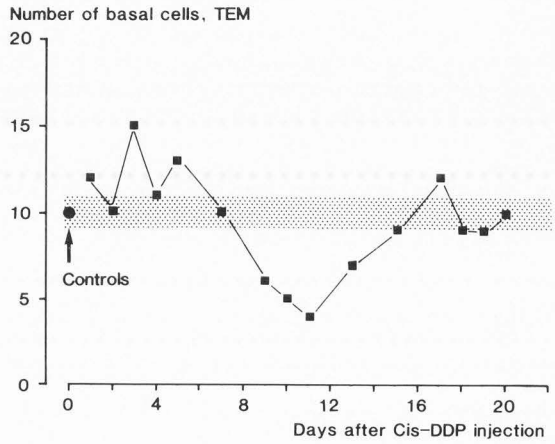


Fig. 9. Number of basal cells as calculated from TEM micrographs.

TEM preparations which were taken each day during the observation period. The results are presented in Fig. 8 and Fig. 9. The basal cell layer in the normal untreated esophageal mucosa consists of about 10 cells. After Cis-DDP treatment the number of basal cells is normal for the first five days. Thereafter, the number is reduced and reaches its lowest value on day 11 (4 cell layers). From day 12, the amount of basal cells steadily increases again and returns to the original value at the end of the observation period. The overall number of cell layers was also calculated on TEM micrographs (Fig. 4). Although the number seems fairly constant, a tendency towards a reduction can be seen in the middle of the observation period around day 7-11, when the height of the mucosal epithelium is at the lowest.

Similarities between Cis-DDP and radiation seem to be the ability to affect clonogenic survival. Single dose irradiation (4.5 Gy) of lymphocytes is shown to result in an increase of the perinuclear spatium with vacuolization, without having any effect on the mitochondria or endoplasmic reticulum (Betz 1974). Therefore, in this paper special attention has been paid to whether or not a similar phenomenon existed after drug injection.

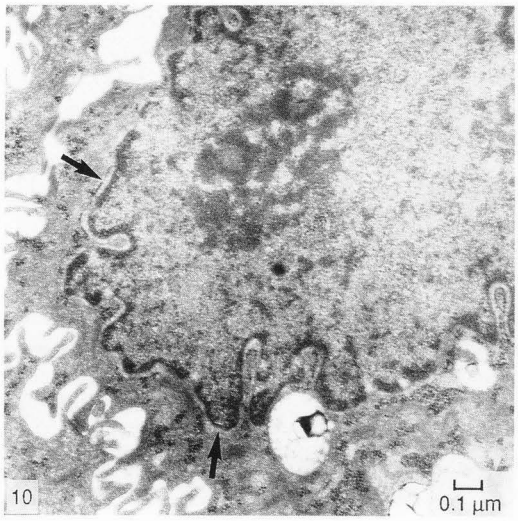


Fig. 10. Nucleus with a perinuclear spatium (arrows).

In the normal esophagus, almost no perinuclear spatium is seen (Fig. 1). The nuclei are normally slightly lobulated with a marked nuclear membrane directly connected to the cytoplasm. One day after drug injection, a perinuclear spatium can be clearly seen (Fig. 10), and was observed however, to a lesser degree until day 11.

Discussion

For squamous cell carcinoma in the head and neck region and the esophagus, better treatment results in terms of local control and survival have been reported when Cis-DDP had been added concomitant with radiation treatment (Shank et al. 1985, Wendt et al. 1987, Seydel et al. 1988). This could either be the result of the radiosensitizing properties of the drug, a cytotoxic effect, or a combination of the two. The interaction between Cis-DDP and ionizing radiation was first described by Richmond and Powers (1976). This effect has been confirmed in other cell systems (Douple and Hoeschele 1978). Earlier publications from this laboratory have described the effects of fractionated radiation alone or in combination with Cis-DDP on the rabbit esophageal mucosa (Albertsson et. al. 1987a, b). Ultrastructural investigations showed damage to the surface area with loosened microridges, increased cell loss and edema. However, towards the end of the observation period which lasted for twenty days, the esophageal mucosa recovered. Within this series, after treatment with Cis-DDP alone and measuring the height of the mucosa with LM and TEM, an increased epithelial height was seen the first days after injection. This may be attributed to an intracellular accumulation of fluid, caused by a cytotoxic effect of the drug, with damage to the mucosal membrane. Like other metals e.g., mercury, Cis-DDP may damage cellular membranes by forming crosslinkages with the sulfhydryl-rich membrane structure itself, and/or inhibit enzyme systems associated with the membrane, thereby producing a leaky membrane phenomenon (Rothstein 1959). The reduction in height and the number of layers of the mucosal epithelium that is observed during the observation period, was maximal around day 11. This finding parallels the reduced amount of basal cells. Cis-DDP probably affects all cells within the epithelial mucosa, but preferably the basal cells. The maximum damage (minimum remaining cells, minimum height of the mucosa) is reached within the same period, i.e., about ten days after injection. The influence of the drug is considered to be maximal shortly after injection (half-life in plasma is about 25-50 minutes, but the plasma clearance has been reported as biphasic with a terminal phase and half-life in the order of days (Litterst et. al. 1973). The drug maintains its neutral structure in the blood, or the extracellular fluid of the body, because of the high chloride ion concentration, that prevents its hydrolysis (Rosenberg 1977). However, within the cell, because of the low chloride ion concentration, it hydrolyzes and form diaquo species which are highly toxic (Aggarwal 1979). Since the biological effect is maximal about day 10, one may assume that within this time range, main parts of the surface layers have loosened and the new cells which have reached the lumen have not started to fall off. The turnover rate fits with this hypothesis (Bertalanffy, 1960). This new cell-layer gradually reaches the loosening period, i.e., it dies off layer by layer. When one compares the height of the epithelial mucosa and number of cell-layers at the end of the observation period, an overshoot is seen. This is a well known phenomenon after radiation treatment and is believed to be the result of synchronized mitotic activity. The primary lesion in the cell due to Cis-DDP is the inhibition of DNA synthesis (Pascoe and Roberts 1974). When counting the number of basal cells in the actual experiment, they are continuously decreasing in number until they reach the lowest value around day 11. This reduction in number could depend on the cytotoxic effects of the drug or a block in the differentiation of the cells. The reduction in number of basal cells is also believed to play a part in the reduction in height of the mucosal epithelium which also reaches its maximum reduction on day 11. Other possible factors could be increased cell loss during the first half of the observation period. An increased cell loss was verified from the scoring of SEM pictures taken at 100X magnification, during the first days after drug injection. The calculation of the overall numbers of cell layers also showed a dip in the middle of the observation period. Towards the end of the observation time, a reactive hyperplasia was seen (Fig. 3). The basal cell layer had recovered (Fig. 9) and seemed intact without any perinuclear spatium. The nuclei were multilobulated with marked nucleoli, and the esophageal mucosa seemed to have been completely restituted.

Conclusion

Cis-DDP exerts a cytotoxic effect on the esophageal mucosa of rabbit. The damage effect is observed already the first day after injection with an intracellular oedema. Thereafter the height of the esophageal epithelium and the basal cell layer steadily decrease to a minimum day 11. This runs parallel to damaged microridges and an increased cell loss as revealed by SEM. At the end of the observation period the esophageal mucosa had completely restituted.

Acknowledgements

This work was supported by the grants from John and Augusta Persson's Foundation for Scientific Medical Research, from the Medical Faculty, Lund University, from Lund's Hospital Research Foundations and from B. Kamprad's Foundation.

This work has been performed at the electron microscopy unit, Faculty of Medicine, Lund University.

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Discussion with Reviewers

Reviewer IV: I always regard a pool of three animals as an absolute minimum for any one time point thereby allowing some kind of statistical analysis to be carried out. I would suggest that the authors group their data, for example, into early time points, intermediate time points, and late time points after treatment.

Authors: We would like to stress that the purpose of our report is not to show statistical significance, but to outline the course of events following adminstration of a given single dose. To investigate three animals per day would have been impractical and unnecessarily time-consuming for the purpose, and to have studied three animals every third day would have been an unsatisfactory way of observing the course of events.

Reviewer IV: Do you think that the absence of sham treatment of control animals could influence the results?

Authors: We have been working with the same animal model system since 1981. The question of a possible influence of anaesthesia was raised at an early stage since it is well known that pentobarbital may effect radiation response. This has been discussed in an earlier paper (Albertsson et al., Scanning Electron Microscopy 1986;III:1117). In a later paper (Albertsson et al., 1987a) Dr. J. Reitan had the same question. We answered that L. Henningsohn had investigated the physiology (beat frequency of the cilia) in ten animals without finding any change due to the anaesthesia. He has now completed these experiments in 24 animals, and the results will be separately published by him soon.