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SCANNING ELECTRON MICROSCOPY OF HUMAN ESOPHAGEAL MUCOSA IN PATIENTS WITH CARCINOMA OF THE ESOPHAGUS

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Abstract

Specimens taken at surgery from 15 patients with carcinoma of the esophagus were examined with scanning electron microscopy. Nine patients were treated with chemotherapy (cisplatin + 5-fluorouracil), surgery and radiotherapy; one received preoperative radiotherapy only; and the remaining five primary surgery only. Scanning electron microscopy was performed on specimens of both tumor tissue and the mucosa at least 5 cm from the tumor. In adjacent non-tumor tissue, damage due to treatment was observed in the form of changes in microridges and increased cell loss. In tumor tissue, the degree of damage was correlated to tumor response to treatment. For patients with no residual tumor after treatment, the ultrastructure was normalized with a low tumor score, while for patients with residual tumor, the score was high.

Key Words: Human esophagus, squamous cell carcinoma, adenocarcinoma, chemotherapy, radiotherapy, surgery, scanning electron microscopy, mucosal epithelium.

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Introduction

In squamous cell carcinoma of the esophagus, earlier treatment strategies of either radiotherapy alone or surgery alone (Earlam and Cunha-Melo, 1980a,b) have given way to multimodal forms of treatment in an attempt to improve the otherwise gloomy prognosis.

At the Department of Oncology, University Hospital, Lund, a phase II trial was carried out during the period 1984-1988, in which patients with esophageal squamous cell carcinoma were pretreated with three courses of cytostatics (cisplatin + 5-fluorouracil) followed by radiotherapy and surgery. Not only was the treatment well tolerated but, as compared with earlier published results, the outcome manifested improvement both in terms of palliation and survival rates (Mercke *et al.*, 1991). In order to intensify treatment, the preoperative radiotherapy was given together with the third course of chemotherapy. The rationale for this derived from findings in an earlier series of animal experiments where rabbits underwent cisplatin treatment and irradiation of the superior mediastinum, and where damage, proliferation and reparative effects in normal tissue were investigated, recovery being found to be better and more rapid in those parts of the trachea and esophagus exposed to the combined treatment than in unexposed areas (Albertsson *et al.*, 1992).

In the present study, scanning electron microscopy (SEM) was performed on a number of patients consecutively treated for esophageal cancer at our department in order to study the effect of treatment both on the tumor and the surrounding mucosa. For comparison, we examined specimens from five patients treated with primary surgery alone. The aim of the study was to assess damage and proliferation in tumor and normal mucosal tissue, and if possible ascertain whether any correlation existed between treatment and clinicopathological outcome variables.

Materials and Methods

The clinical characteristics of the 15 patients with esophageal cancer, either squamous cell carcinoma (n = 13) or adenocarcinoma (n = 2), are shown in Table 1.

The patients were divided into three groups, according to their treatment:

1) Control group (n = 5, 1 female, 4 males), aged 61-86 years who underwent primary surgery without any pretreatment.

2) Standard treatment group (n = 7, all males), aged 60-68 years, treated with three cycles of chemotherapy and preoperative radiotherapy (24 Gy).

Group **2a**) Standard treatment, no residual tumor.

Group **2b**) Standard treatment, residual tumor.

3) Pretreated group (n = 3, 2 females, 1 male), aged 66-76 years, whose treatment deviated from that of the standard treatment group for one reason or another.

Sampling

Specimens for SEM were taken in conjunction with surgery. One specimen was taken from the tumor area (TA); if no residual tumor was visible at surgery, a specimen was taken from the area initially considered to be tumor-involved. In addition, for control purposes, a non-tumor area (NTA) specimen was taken from the esophageal mucosa at least 5 cm from the visible border of the tumor area.

Treatment

The treatment protocol consisted of three courses of chemotherapy followed by radiotherapy and surgery:

Chemotherapy Chemotherapy consisted of cisplatin [90-120 mg/m^2 BSA (body surface area)] on day 1, and 5-fluorouracil (5-FU 1000 mg/m^2 BSA) daily in continuous infusion on days 1-5. Before cisplatin administration, the patients were prehydrated with 1000 ml 0.9% saline given as a 2-hour infusion. Cisplatin was dissolved in 2000 ml 0.9% saline and given together with 500 ml 15% mannitol as an intravenous infusion over four hours. Uresis was measured every fourth hour, diuretics being given if it was less than 400 ml for the 4-hour period. Treatment with 5-FU started immediately after completion of the cisplatin infusion, the 5-FU being dissolved in 2000 ml 0.9% saline and given as continuous 24-hour infusion for five consecutive days.

Radiotherapy For patients without metastasis, an absorbed dose of 64 Gy to the esophageal tumor (target volume I) was planned in two series as pre- and postoperative radiotherapy. In all cases, radiation therapy was given with 6 MV or 8 MV photons, using a linear accelerator. Target volume I was defined as the tumor demonstrated at chest radiography or CT (computerized tomography). For tumors at or above the tracheal carina, the caudal border of target volume I was set 5 cm below the lower extension of the tumor, whereas its cranial border included the supraclavicular nodes. For these upper tumors, a 3-field approach was used both pre- and postoperatively. For tumors, the bulk of which was located below the level of the carina, the cranial border was set to include 5 cm of radiographically uninvolved esophagus, whereas the celiac lymph nodes were included in target volume II, and defined the caudal border of target volume I.

Any affected nodes in the celiac region were resected at surgery. If histopathologic examination showed the presence of viable malignant cells, the total absorbed dose was 40 Gy, otherwise only 24 Gy (i.e., only the preoperative radiotherapy was given). In the case of tumors below the tracheal carina, AP-PA (anteroposterior-posteroanterior) fields were used preoperatively (target volumes I and II); and postoperatively a 3-field technique with one dorsal and two oblique portals was used for target volume I and AP-PA fields for target volume II with a specified dose of 40 Gy. The target-absorbed dose was specified according to minimum absorbed dose. Daily fractionated radiation was given with a target dose of 2.0 Gy.

Surgery Surgery included laparotomy for inspection of liver and celiac nodes, the latter being resected if cancer was suspected. The stomach and the duodenum were mobilized and pyloromyotomy performed. The esophagus was resected through a right-sided thoracotomy, the stomach being pulled up into the chest and an anastomosis performed between the fundus and the proximal esophagus.

Specimen preparation for SEM

Specimens for SEM were fixed in 2.5% glutaraldehyde (in 0.15 M cacodylate buffer, pH = 7.3) for 12 hours, followed by postfixation in 1% osmium tetroxide in 0.15 M cacodylate buffer for two hours. After dehydration in a graded ethanol series and critical point drying, the specimens were sputter-coated with gold and examined in a Philips 515 SEM operated at 20 kV.

Scoring system

For evaluation of the specimens, the following 5-point scoring system was used:

Score 0: Normal epithelium.
Score 1: Membrane damage with edema and exudate.
Score 2: Damaged microridges manifesting the 'farcimen phenomenon' (i.e., broken up into short segments linked like a string of sausages), nodules (knobs), fluid-containing vesicles (blebs) and other protrusions.
Score 3: Ulceration, microridges rudimentary or lost.
Score 4: Manifest destruction, no normal surface structures identifiable.

From each specimen, at least ten different areas were evaluated by five independent observers. Corresponding tumor and non-tumor specimens were rated according to this scoring system, yielding a mean case score and mean group score (see Fig. 8, for results).

Results

Group 1. Control group

SEM of esophageal mucosa specimens from the control group of patients undergoing no pretreatment (Table 1) showed polygonal cell flakes, arranged in regular patterns (Figures 1a-c). Cell borders were clearly identifiable, and did not protrude from the surface of the mucosa. Some cell loss was usually seen,

Table 1. Clinical data from 15 patients with esophageal carcinoma.

No.	Age	Sex	Level	Pathol	Pretreatment	Survival months	Score SEM Tumor area	Score SEM Non-tumor area	Figures
Group 1									
1	75	M	Low	S	--	7			1a, 1b, 1c
2	86	F	Low	S	--	11			
3	82	M	Low	A	--	5	4.0	1.37	2a, 2b, 2c 3a, 3b, 3c
4	62	M	--	S	--	5			
5	61	M	Low	A	--	7			
Group 2a (standard treatment, no residual tumor)									
6	64	M	Low	S	3 (cht) + RT 24 Gy	5			
7	62	M	Low	S	3 (cht) + RT 24 Gy	24	1.5	1.12	4a-4e
Group 2b (standard treatment, residual tumor)									
8	65	M	Mid	S	3 (cht) + RT 24 Gy	9			
9	60	M	Mid	S	3 (cht) + RT 24 Gy	29			
10	60	M	Low	S	3 (cht) + RT 24 Gy	40	3.0	2.0	5a, 5b
11	68	M	Up	S	3 (cht) + RT 24 Gy	12			6a, 6b
12	61	M	Low	S	3 (cht) + RT 24 Gy	26			
Group 3									
13	66	F	Low	S	1 (cht) + RT 40 Gy	16			
14	68	F	Low	S	1 (cht) + RT 24 Gy	7	3.5	2.0	
15	76	M	Mid	S	RT 40 Gy	4			7a, 7b

S = Squamous cell carcinoma; A = Adenocarcinoma; cht = chemotherapy; RT = radiotherapy.

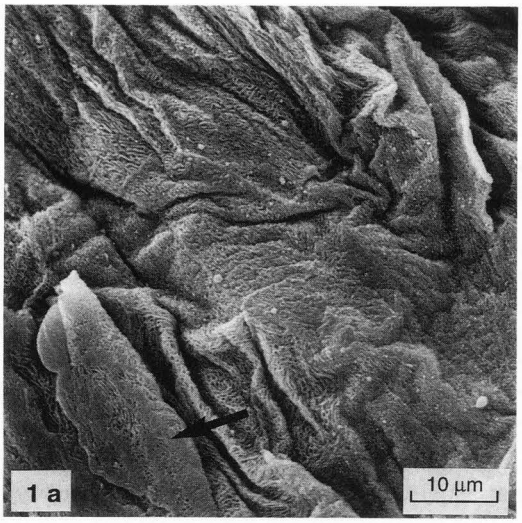
often occurring in flakes of groups of cells, but sometimes of whole single cells. Cell surfaces were covered with microridges, often parallel to each other but sometimes in whorled or convoluted configurations, the patterns being homogeneous within a given cell but often varying from one cell to another. The microridges usually manifested small, barely discernible nodular irregularities (Fig. 1b). Occasionally the microridges appeared to be swollen, manifesting the 'farcimen phenomenon' (Fig. 1c). Sometimes the nodular irregularities appeared to be distended, like small bullae, connected to the original microridges by fine 'stems'; and sometimes they had the form of a broad-based protrusions (Fig. 2c). Occasionally microridges covered the greater part of the cell surface in the form of protrusions (Fig. 2b), and sometimes they were grouped in nidulate configurations (Fig. 2a). Two of the patients in this group were found to have adenocarcinoma. The TA specimen of one of the two patients with adenocarcinoma manifested polygonal cells outlined with short, stubby microvilli (Fig. 3a). Occasionally a closed packed rim of microvilli could be seen surrounding a central area of mucus. In another area, the cells manifested marked bulging and their surfaces were covered with microvilli (Fig. 3b). For comparison, Fig. 3c shows the normal squamous epithelium in an NTA specimen from the same patient.

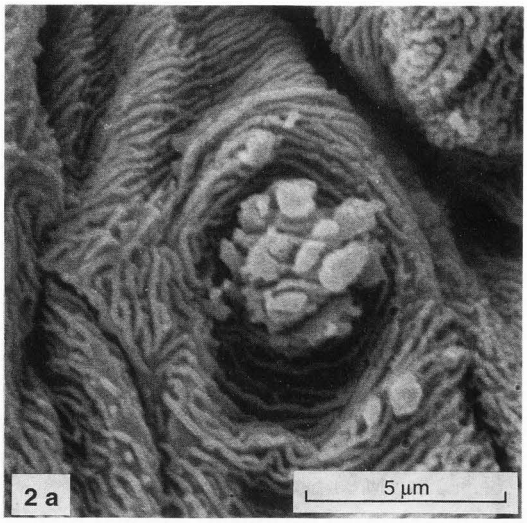
Group 2a (Standard treatment, patients with no residual tumor after preoperative treatment, n = 2)

No malignant cells were identifiable within the original tumor area, specimens being similar to those from normal epithelium (NTA), though some differences could be observed. In TA specimens, cells were rounded and plaque-like, lying loosely on the surface (Fig. 4a), without the interconnections seen between normal cells. In some areas the cells were swollen with edema and exudate (Fig. 4b); in others, the microridges were arranged in irregular configurations, being sometimes very closely packed, and manifesting the 'farcimen phenomenon' (Fig. 4c). Intercellular spaces took the form of cracks and fissures. No microorganisms were found. Figures 4d and 4e from the same patient present micrographs from non-tumor area. The cell borders are clearly defined (Fig. 4d) and microridges are arranged in regular patterns.

Group 2b (Standard treatment, patients with residual tumor)

TA specimens manifested pronounced heterogeneity in cell surface morphology. In some areas, no normal microridge patterns could be seen; some areas were completely denuded of microridges, and in other areas, they were present only in rudimentary form (Fig. 5a).


1 a
10 µm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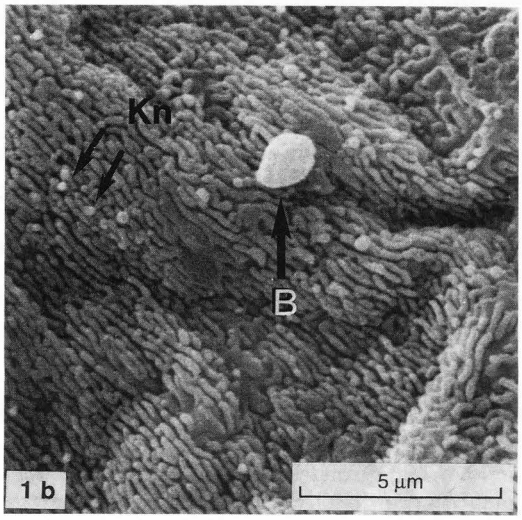
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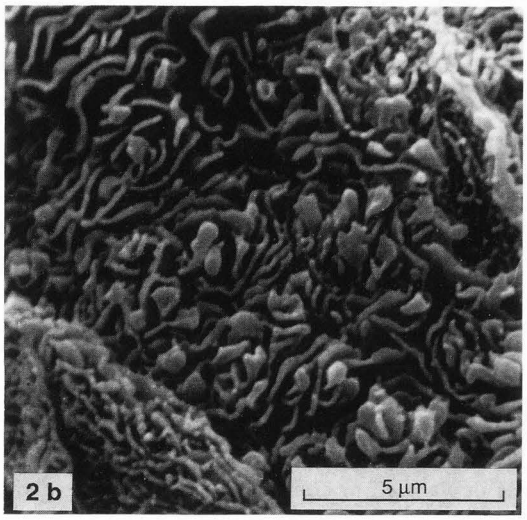
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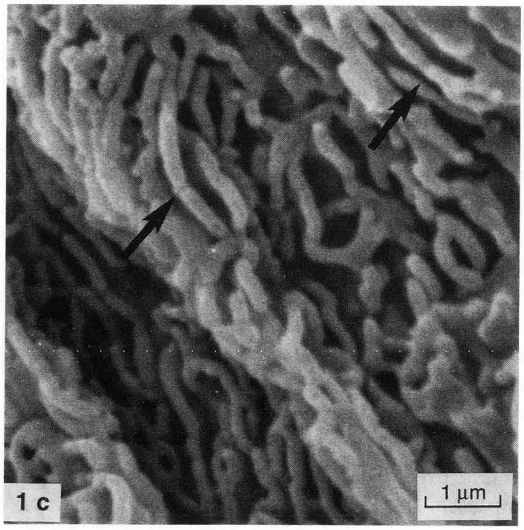

1 c
1 µm

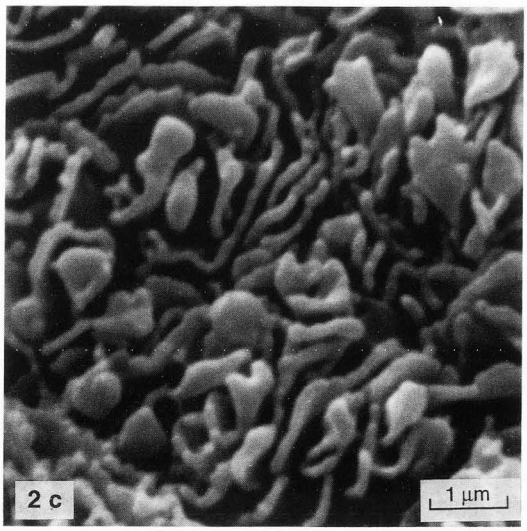

2 c
1 µm

Figure 1 (facing page, left). Scanning electron micrographs of a non-tumor area (NTA) specimen from a group 1 (non-pretreated) patient (no. 1) with squamous cell carcinoma, showing: **a**) wrinkled epithelium, and exudate (arrow); **b**) mucus-containing bulges (arrow, B) and knobs from microridges (arrow, Kn); **c**) swollen microridges manifesting the 'farcimen phenomenon' (arrow) and cell borders raised in relief.

Figure 2 (facing page, right). Micrographs of an NTA specimen from a group 1 (non-pretreated) patient (no. 3) with adenocarcinoma, showing: **a**) clustered protrusions contained within a nidulate arrangement of microridges; **b**) protrusions; and **c**) central area of Fig. 2b, at a higher magnification.

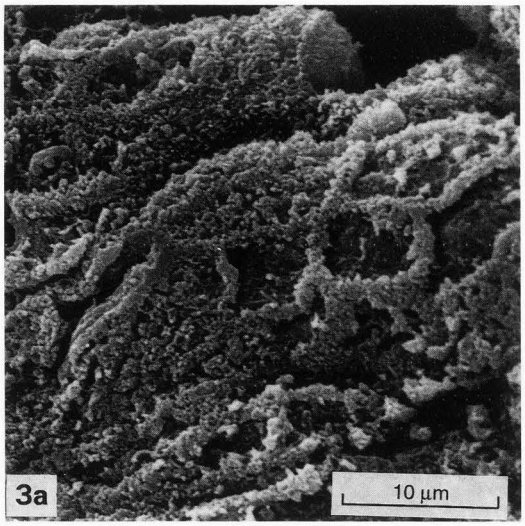
Figure 3 (at right). Micrographs of specimens from a group 1 (non-pretreated) patient (no. 3) with adenocarcinoma: **a**) TA specimen showing cells with borders covered with microvilli; **b**) TA specimen, showing singular bulging cells, the entire surface of which is covered with microvilli; **c**) NTA specimen, showing swollen cell borders and microridges in regular arrangements.

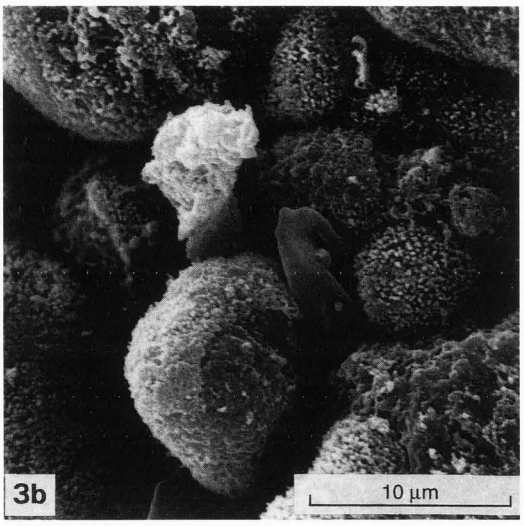
In the NTA specimen, cell borders were distinct and raised in relief, the cell surface manifesting regular patterns of microridges (Fig. 5b). Microridges manifested great morphological variation from one area of a given cell to another, and there was no sign of the regular surface pattern seen in normal cells. Normal cell borders were absent, intercellular spaces taking the form of cracks and fissures. In some cases, abnormalities of cell surface morphology were manifested, with no normal features remaining (cf. Figures 6a and 6b). In TA specimens, cells manifested gross superficial destruction, with filamentous processes extending across the surface (Fig. 6a); cell borders could not be discerned. In NTA specimens, the surface was covered with short, stubby microridges of a nodular appearance (Fig. 6b), which could be verified by transmission electron microscopy (TEM).

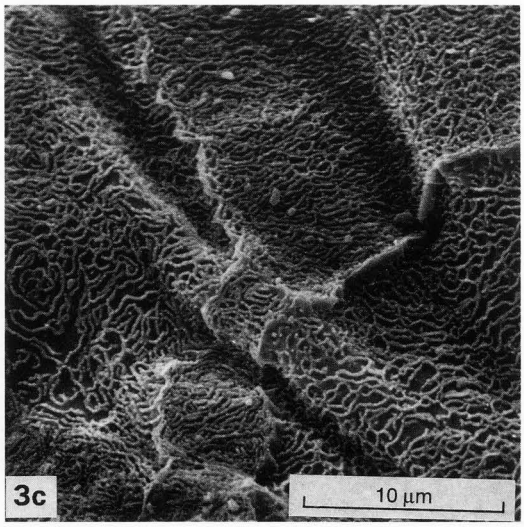
Group 3

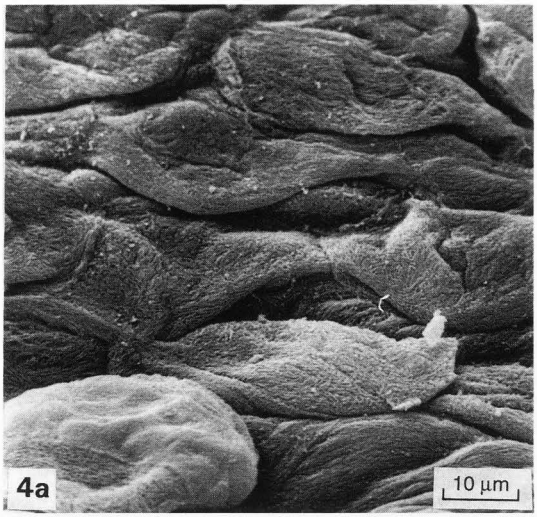
For patient number 15, given preoperative radiotherapy (40 Gy) but no chemotherapy, the TA specimen manifested gross superficial destruction, and no normal cell structures could be discerned (Fig. 7a). His NTA specimen showed slight treatment damage, manifesting edema, exudate and a somewhat wrinkled epithelium (Fig. 7b).

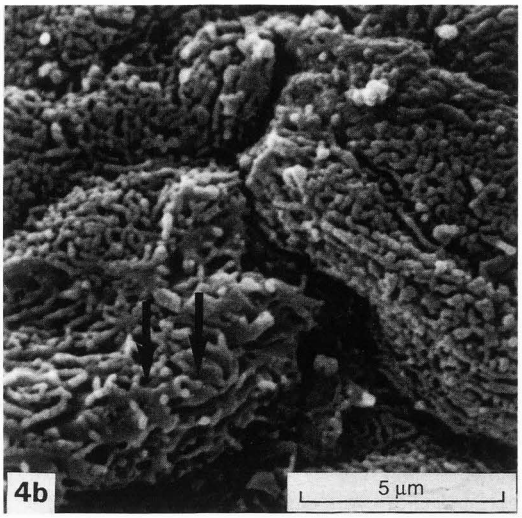
From the scoring results shown in Fig. 8, it can be seen that for group 1 (i.e., patients who underwent surgery only) the NTA score was just over 1, and the TA score 4. For patients with no residual tumor after preoperative treatment (n = 2), the TA score was 1.5 and the NTA score 1.1. For patients with residual tumor at surgery, the NTA score (2.0) was higher than in the other two groups, and the TA score was markedly higher, 3.0 in group 2b and 3.5 in group 3.

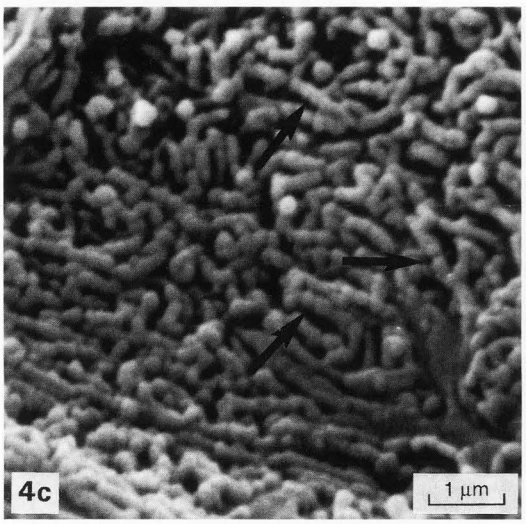


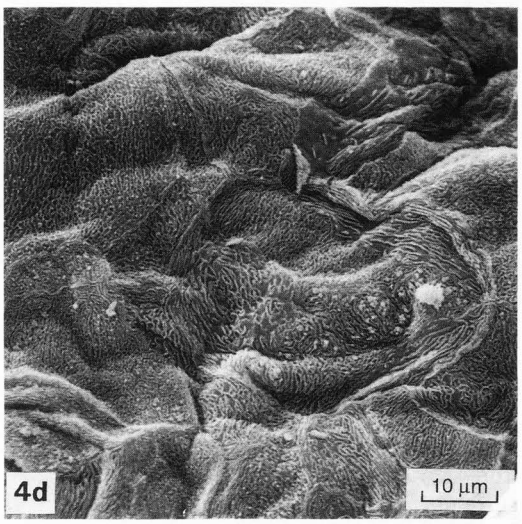












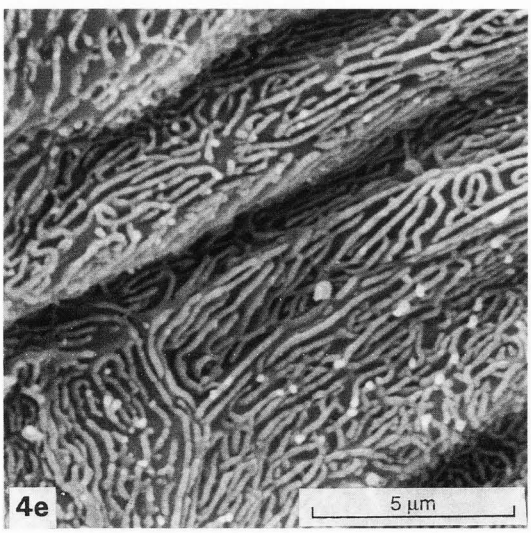
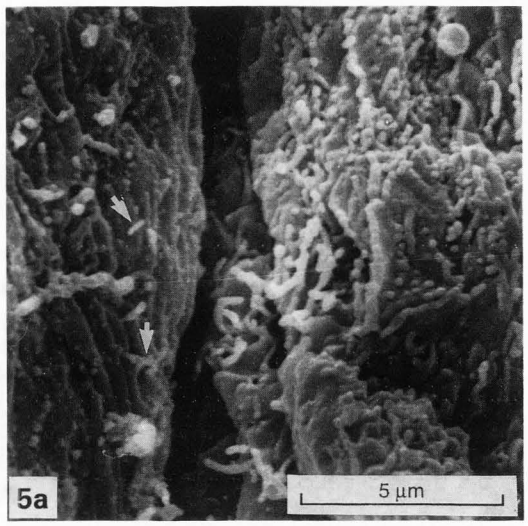
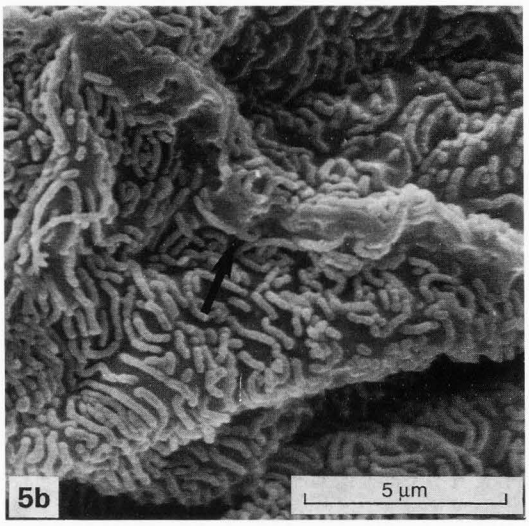
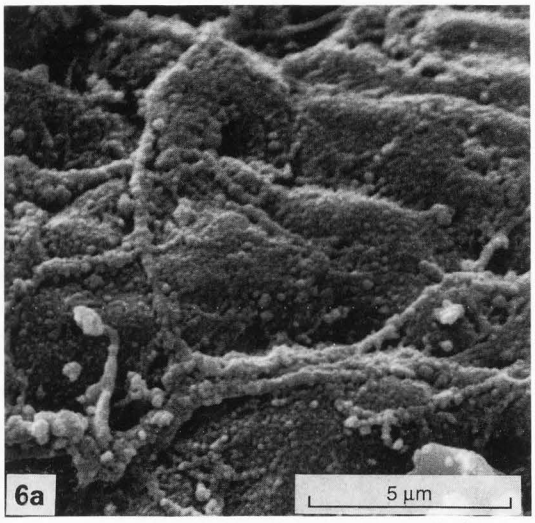


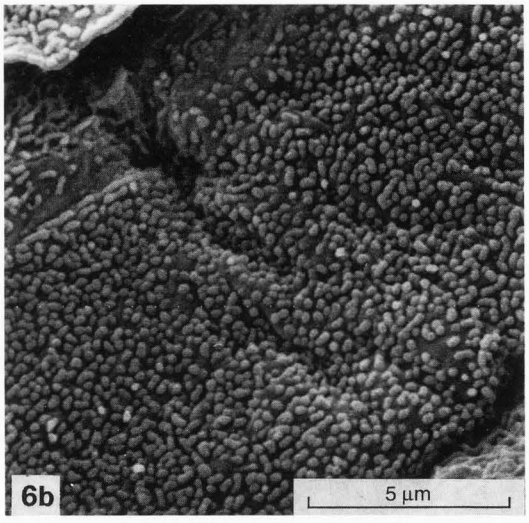
Figure 4. Micrographs of specimens from a group 2a (pretreated) patient (no. 7) with no residual tumor at surgery: **a**) TA specimen showing plaque-like epithelial cells in singular formations; **b**) TA specimen showing swollen cells with disrupted microridges, edema and exudate (arrow), the microridge abnormality presumably being attributable to treatment; **c**) an area from Fig. 3b at a higher magnification, showing densely packed microridges with the farcimen phenomenon (arrows), and microridges in irregular patterns; **d** and **e**) NTA specimen showing normal microridges in regular patterns and clearly defined cell borders.

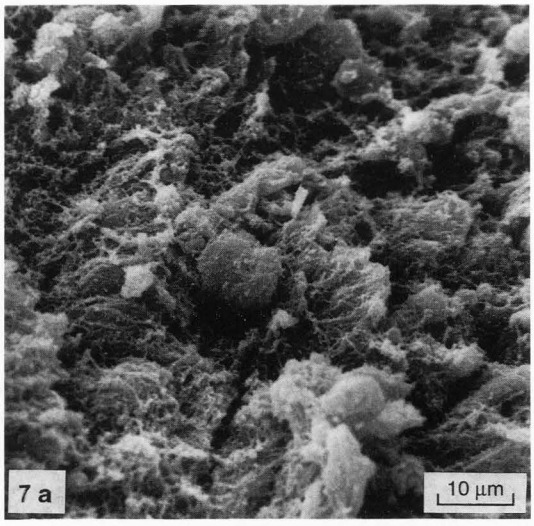
Figures 5 and 6 (on facing page). Micrographs of specimens from group 2b (pretreated) patients (no. 10, **Figure 5**; and no. 11, **Figure 6**) with residual tumor at surgery: **Fig. 5a**) TA specimen showing cells with microridges almost absent or only present in rudimentary form (arrows); **Fig. 5b**) NTA specimen showing slightly damaged microridges, and cell borders with exudate (arrow); **Fig. 6a**) TA specimen, showing heavy surface destruction; and **Fig. 6b**) NTA specimen, showing the surface covered with short, stubby microridges.











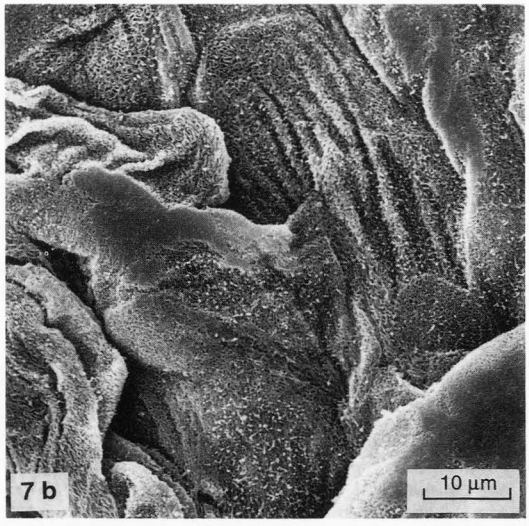


Figure 7. Micrographs of specimens from a group 3 (non-standard pretreatment) patient (no. 15) with residual tumor at surgery: **a**) TA specimen, showing heavy surface destruction; **b**) NTA specimen, showing wrinkled epithelium, and exudate.

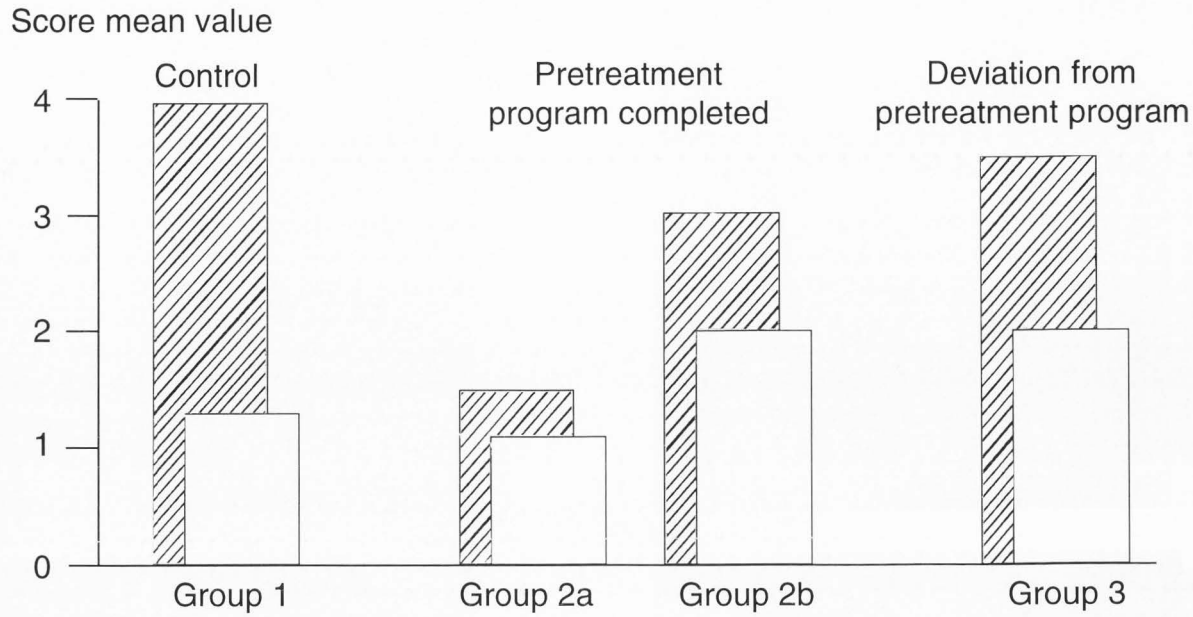


Figure 8. Mean scores for tumor area (hatched) and non tumor area (unhatched) specimens in groups 1, 2 and 3.

Discussion

It has been a time-honored axiom in cancer therapy that the two principal treatment modalities, chemotherapy and radiotherapy, should not be given simultaneously but staggered to avoid unacceptable high toxicity in normal tissue. However, in squamous cell carcinoma of the esophagus, earlier treatment strategies of either radiotherapy alone or surgery alone (Earlam and Cunha-Melo, 1980a,b) have given way to multimodal forms of treatment comprising chemotherapy, radiotherapy and surgery (Launois *et al.*, 1981; EORTC, 1985; Kelsen 1985; Carey *et al.*, 1986; Popp *et al.*, 1986; Leichman *et al.*, 1987; Hambraeus *et al.*, 1988; Forastiere *et al.*, 1990; Herskovic *et al.*, 1992), an approach introduced with a view to improving both local control and the otherwise gloomy prognosis associated with this disease.

The recently reported treatment of esophageal cancer with three cycles of chemotherapy combined with radiotherapy and surgery has yielded promising results (Mercke *et al.*, 1991). In the present study, specimens from 15 patients were examined by SEM to obtain further information. Specimens from five of the patients, who had undergone surgery only (without pretreatment), were selected for use as controls; however, their NTA specimens were not found to be completely normal according to previously published morphological data (Ackerman *et al.*, 1976; Robinson *et al.*, 1981). In those studies, normal esophageal mucosa is described as a flat surface overlaid with polygonal epithelial cells and regular patterns of microridges.

In the present patients, the surface adjacent to the tumor area was wrinkled or folded, and the microridges were not completely normal but manifested protrusions of various kinds and sizes. A fairly common finding was the presence of small nodular irregularities of the membrane that apparently developed, becoming distended either like small balloons and attached to the microridges by stem-like processes, or in the form of a broad based protrusions arranged in structured groups or covering a portion of the surface area (Figures 2a-c). Although at first glance it might seen reasonable to interpret these changes in microridge morphology as damage attributable to treatment (e.g., chemotherapy or radiotherapy), they were also seen in untreated cases and may well constitute a non-specific degenerative process associated with any of a variety of factors.

The specimen shown in Fig. 2 is from the esophageal mucosa of an 82 year old man with adenocarcinoma of the esophagus who underwent primary surgery only (i.e., no pretreatment with chemotherapy or radiotherapy); in this case, the microridge degeneration may constitute, wholly or partly, an expression of the normal ageing process. Moreover, as patients treated for esophagus cancer commonly manifest a weight loss of 5-15 kg (11-33 lbs) at diagnosis, and often loose more weight during treatment, it is not impossible that this weight loss and dehydration also affect the esophageal mucosa, perhaps explaining the wrinkled superficial appearance.

Ackerman *et al.* (1976) reported that the superficial microridges seen in SEM correlate well with the short cytoplasmic processes of superficial mucosal cells seen in TEM. The microridges are believed to be of functional importance in maintaining epithelial integrity, cellular contact being enhanced by interdigitation. An early observable change is that the microridges swell, increasing in diameter, and manifesting what we have termed the 'farcimen phenomenon' (i.e., the microridge is broken up into short segments, resembling a string of sausages). A similar phenomenon is seen in very small blood vessels exposed to radiation, and is said to be due to the swelling of endothelial cells. Microridges have no

connection to endothelial cells, and it is not known whether there is any cytoplasmic content. However, they form abundant connections (desmosomes) with overlying or underlying cell layers.

In those group 2a cases, where no residual tumor was found at surgery (n = 2), the esophageal mucosa in the original tumor area was normalized, as reflected in the mean scores for these patients (Fig. 8) which were very close to the NTA scores for group 1 patients who underwent surgery only (i.e., no chemotherapy or radiotherapy pretreatment).

All the normal structures can be identified in TA specimens (Figures 4a-c). An abnormal finding was that of loose cells on the cell surface, which may be attributable to an effect of the treatment on desmosomes, an issue that we shall be investigating in further studies using TEM.

Group 2b patients, who had undergone pretreatment but manifested residual tumor at surgery (Figures 5a 5b, 6a and 6b), had higher mean scores both in TA and NTA specimens (Fig. 8). Although this finding is striking, it should be borne in mind that the series is too small to allow any firm conclusions to be drawn, other than that perhaps group 2b patients constitute worse cases generally, something that equally applies to group 3 patients (Figures 7a and 7b).

Concerning the abnormal morphology of tumor cells, with microridges absent or only present in rudimentary form, the presence of poorly defined microridges has previously been reported both in reflux esophagitis (Dilly and Mallinson, 1975) and in preneoplastic and neoplastic lesions (Williams *et al.*, 1973). Moreover, both the reduction in the number of microridges and their less regular arrangement, as seen on malignant esophageal cells in the present study, have been described previously and interpreted as possibly due to the rapid growth and turnover of malignant cells (Siew and Goldstein, 1981). In the present study, no correlation was found between tumor score and survival.

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Discussion with Reviewers

S. Siew: You state that in cases with no visible residual tumor, specimens were taken from the area initially considered to be tumor involved. By what means had such areas been identified to have been involved prior to therapy?
Authors: All patients were thoroughly investigated and pretherapy staging was based on clinical history and examination, barium radiography of esophagus, esophagoscopy, chest radiography, CT of thorax and upper abdomen, blood count and serum tests of liver function. All these examinations were repeated after chemotherapy and radiotherapy for evaluation of response.

S. Siew: Did you examine the tissues by means of light microscopy? If so, what was the correlation between the histopathologic and scanning electron microscopic findings? More particularly, in the 2 cases with no visible gross tumor, was there evidence of tumor on microscopic examination?
Authors: Immediately after surgery, all the tissues were examined with light microscopy, and also later on with TEM, results of which will be presented at a later date. In the 2 cases with no visible gross tumor, there was no evidence of tumor on light microscopic examination.

S. Siew: You have shown that there was no correlation between the tumor score and survival (Table 1). However, other factors have to be taken into consideration such as the fact that esophageal tumors often spread beneath the mucosa. In such cases, the overlying mucosa would have a spuriously normal appearing surface. Further, depth of invasion and metastasis, regional and distant, determine the length of survival.
Authors: Your are absolutely correct and for this reason, all patients were thoroughly examined (see answer to your first question above). Other investigations have indicated that a complete response to chemotherapy is strongly associated with a prolonged disease-free survival (Rooney *et al.*, 1985; Al Kourainy *et al.*, 1987; Jacobs *et al.*, 1987; Thomas *et al.*, 1988). Unfortunately in our study, the group of patients (n = 2) with no residual tumor after treatment died in intercurrent deaths. One of the patients died after five months in pulmonary embolism and autopsy showed no tumor. Also the other patient with no residual tumor after pretreatment, died in intercurrent death and again autopsy showed no residual tumor.

S. Siew: There were 2 cases of adenocarcinoma. Did they show similar findings? Your micrographs (Figures 2a-c and 3a-c) are from one and the same case: Figures 2a-c and 3c being of non-tumor affected tissue and Figures 3a and 3b from the tumor. Do you not consider that the appearance of Figures 3a and 3b, with the presence of microvilli and "bulging" cells, is in keeping with that of a poorly differentiated adenocarcinoma and that the bulging of the cells is due to the fact that they are columnar?
Authors: Yes, the findings were similar in the two cases with adenocarcinoma and I find your interpretation of the micrographs interesting and absolutely right.

S. Siew: As Barrett's esophagus is an important predisposing factor of esophageal carcinoma, did you see evidence of that in your cases?
Authors: Barrett's esophagus is an important predisposing factor only for adenocarcinoma and we did have two cases of adenocarcinoma in this study; we did find evidence of Barrett's esophagus in one of these cases.

S. Siew: Figure 7a illustrates the SEM findings in a patient who had residual tumor after one course of radiotherapy. Are you able to differentiate the degree of damage inflicted by radiotherapy in the production of the heavy surface destruction?
Authors: Unfortunately we could not differentiate the damage. However, the exudation on the surface and the fibrin-like network could be associated to radiation damage.

M.J.A. Cornelissen: Since you mention in your **Introduction** that the aim of the study is to assess proliferation, would it not be appropriate to have an estimation of proliferation capacity after treatment by using proliferation markers. Please comment and give some suggestions.
Authors: You are absolutely correct. In future, we are planning to investigate the proliferation capacity for the tumors with BuDR before, and also after, treatment.

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