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Radiation-induced increase in hyaluronan and fibronectin in bronchoalveolar lavage fluid from breast cancer patients is suppressed by smoking

L. Bjermer*, R. Hällgren**, K. Nilsson*, L. Franzen***, T. Sandström+, B. Särnstrand++, R. Henriksson***

Radiation-induced increase in hyaluronan and fibronectin in bronchoalveolar lavage fluid from breast cancer patients is suppressed by smoking. L. Bjermer, R. Hällgren, K. Nilsson, L. Franzen, T. Sandström, B. Särnstrand, R. Henriksson.

ABSTRACT: Bronchoalveolar lavage (BAL) fluid was analysed from 21 patients with breast cancer, stage $T_1N_0M_0$, who had undergone tumour resection and post-operative local irradiation (accumulated dose 56 Gy). The lavage was performed two months after radiotherapy, in the anterior part of the lingula (left side) or of the right middle lobe (right side), depending on which side had been exposed to radiation.

The patients had significantly increased concentrations of fibronectin (FN) ($p<0.001$), hyaluronan (HA) ($p<0.01$) and albumin ($p<0.05$) in BAL fluid compared with the healthy controls (n=19). However, when the patients were separated, according to smoking history, it was obvious that the inflammatory reaction occurred entirely in the nonsmoking patient group (n=10), whilst no difference could be found between the smoking patients (n=11) and the controls. In the nonsmoking patient group, there was a sevenfold increase in BAL concentrations of FN and a threefold increase in HA. Moreover, four patients had detectable levels of procollagen III peptide in BAL, all were nonsmokers. The smoking habits of the controls had no influence on the BAL measurements.

These findings indicate that smoking interferes with the radiation-induced early inflammatory connective tissue reaction of the lung. Finally, the results justify further investigation of interaction of smoking with cancer treatment, both from the view of therapy effectiveness and reduction of adverse effects.

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Depts of * Lung Medicine and *** Oncology, University Hospital, Umeå, Sweden. ** Dept of Internal Medicine, University Hospital Uppsala, Sweden. + National Institute of Occupational Health, Medical Division, Umeå, Sweden. ++ Draco Pharmaceuticals, Lund, Sweden.

Correspondence: L. Bjermer
Dept of Lung Medicine
University Hospital
S-901 85 Umeå
SWEDEN

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Radiation-induced pneumonitis and lung fibrosis are important clinical problems [1, 2]. The effects of irradiation on normal lung tissue have been extensively studied in animals, whilst the effect of irradiation on the human lung has been mainly elucidated using lung physiology and chest X-ray [1, 3]. Since bronchoalveolar lavage (BAL) was introduced into the field of lung research, this technique has become a valuable tool for the investigation of various interstitial lung diseases [4]. Previous BAL studies on radiation-induced injury in man have been performed on patients with malignancies within the lung [5]. The interpretation of these results was hampered due to the difficulty of separating the alveolar reaction induced by the radiation from the reaction induced by the tumour itself.

In the present study, we have investigated a group of patients with breast cancer, stage $T_1N_0M_0$, who, after breast-saving removal of the tumour, underwent post-operative radiation therapy against the breast. This form of radiation therapy, unfortunately, also involves the underlying lung tissue as revealed by physiological and radiological measurements [1]. We have tried to evaluate the connective tissue response of the lung by measuring hyaluronan (HA) (hyaluronic acid or hyaluronate by older nomenclature) and procollagen III peptide (PIIIP) in the BAL fluid. Previous experimental and clinical studies have shown that these substances are quite sensitive markers of the matrix repair process occurring subsequent to inflammatory damage of the lung [6–8]. Analysis of fibronectin (FN) was also included in the study, since increased BAL concentrations of this product, partly derived from alveolar macrophages, have been reported in interstitial fibrosing lung diseases and after radiation-induced lung damage [9, 10].

Another objective of the present study was to investigate whether smoking has an effect on the connective tissue response to the radiation therapy. The present results show that smoking patients are less prone to develop a radiation-induced pulmonary reaction than nonsmoking patients.

Patients and methods

Twenty one consecutive patients, mean age 48 yrs, range 35–64 yrs, with unilateral breast cancer, stage $T_1N_0M_0$, were studied. Eleven were smokers and 10 lifelong nonsmokers. After resection of the tumour, postoperative local radiation therapy (2 Gy·day^{-1}; photon beams 6 MV) was given five times a week, for 5.5 weeks. The total accumulated mean target dose was 56 Gy (fig 1). The patients were investigated by BAL two months after the end of the radiation therapy. The control group included 19 healthy members of the medical staff and students (11 males, 8 females), 7 smokers and 12 nonsmokers; mean age 35 yrs, range 16–57 yrs. All smokers reported a daily consumption of 10–20 cigarettes and all had been smokers for more than one year prior to the study. The study was approved by the local Ethics committee. All patients and controls who participated in the study were informed and gave their consent.

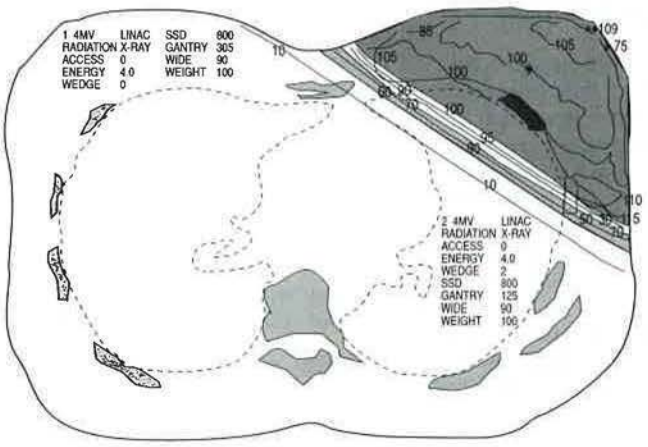

Fig. 1. – A typical dose plan for postoperative radiation of breast cancer following surgery. Note that lung parenchyma falls within the dose range of 90%.

Bronchoalveolar lavage

BAL was performed as described previously [6]. A total of 240 ml sterile Krebs' Ringer phosphate buffer (pH 7.3) at 37°C was infused in boluses of 60 ml. Depending on the side of the breast irradiated, BAL was performed either in the anterior part of the lingula (left side) or in the anterior part of the middle lobe (right side). The mean recovery of fluid instilled was 124±18 (SD) ml, with no significant differences between patients and controls. The recovery did not differ between the right and left sides of the lung. The lavage fluid was kept on ice and filtered through a nylon filter (pore diameter 100 μm).

Cell preparation and analysis

The collection of cells and the preparations for cytological analysis were made as described previously [6]. The cytocentrifuge preparations were stained according to May-Grünwald/Giemsa before differential counting.

Hyaluronan. HA was analysed in BAL and serum duplicate samples by a radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden) adopted according to the principles described previously [7]. The detection limit for HA is 5 μg·l^{-1} by this method. Variability of the measurements of HA was <10%. The mean serum HA values for the patient group was 25±19 (SD) μg·l^{-1} with no significant differences among the patients or between the patient group and the age-matched controls.

Procollagen III peptide. [PIIIP] was analysed using a radioimmunoassay (Boehringer-Ingelheim). The detection limit for PIIIP was 0.2 μg·l^{-1}. Variability of the measurements was <10%.

Fibronectin. FN was analysed with a double-sandwich enzyme-linked immunosorbent assay (ELISA) technique developed in our laboratory. Microtitre plates (NUNC, Denmark) were coated with rabbit-antihuman FN antibodies (Dakopatts, Denmark) diluted 1:2000 in carbonate buffer, pH 9.6. After incubation at room temperature for 24 h the titre plates were carefully washed. Phosphate buffered saline (PBS) containing 0.05% albumin was added and the plates were left at room temperature for one hour. The PBS was removed, BAL fluid and serum samples were added in a diluted series together with horseradish peroxidase–labelled antihuman FN 1:2000 (Dakopatts, Denmark) as the second antibody and the plates were incubated for 90 min at room temperature. The amount of bound peroxidase, which was proportional to the amount of FN in the sample, was measured by analysing the enzymatic activity on ortho-phenylendiamine. Plasma fibronectin of nephelometric quality was provided by Sigma Chemicals and used as a standard. The detection limit was 10 μg·l^{-1}. Intra- and interassay variation was <7%.

Chest X-ray and calculation of irradiated volume

Chest X-ray was performed with an anterior and lateral view combined with a 45° rotation (oblique) picture on the irradiated side. This projection made it possible to study the anterolateral part of the lung close behind the irradiated breast. By computerized tomographic (CT)-scanning the volume of lung parenchyma falling within the dose range of 90% was calculated and used as a border limit for estimation of the amount of lung tissue involved in the target field (fig. 1).

Measured BAL substances and cellular data were log-transformed. Log data followed a normal distribution. The Wilcoxon's non-parametric rank sum test was used for statistical analyses. Correlation coefficients were calculated by the Spearman's rank correlation test.

Results

Chest X-ray and irradiated lung volume

Signs of pneumonitis on chest X-ray were seen in three patients. All were nonsmokers. The calculated amount of lung involved in the radiation field (>90% of target dose), was 66 ml (range 27–110 ml) in the chest X-ray "positive" group compared to 104 ml (range 27–253ml) in the "negative" group.

Bronchoalveolar lavage analysis

The BAL findings after postoperative radiation therapy of patients with breast cancer (n=21) are summarized in table 1. The smoking habits of the patients had a significant influence on concentrations of the measured substances (fig. 2 and table 1). The nonsmoking patients had a sevenfold increase in their mean FN concentration in BAL fluid compared with the healthy nonsmoking controls ($p<0.001$). The HA concentrations in BAL fluid were also significantly increased in this patient group ($p<0.001$). The lavage recovery of albumin was also significantly increased in the nonsmoking patient group ($p<0.01$). In contrast, the smoking patients had similar lavage findings to the controls (table 1), and nonsmokers and smokers among the controls had similar lavage concentrations of fibronectin, HA and albumin (table 1). The PIIIP concentrations in BAL were below the detection limit in the controls but measurable amounts were found in four of the patients (range 0.3–1 μg), all were nonsmokers. Three of the patients who showed signs of pneumonitis on the chest X-ray, and who were also nonsmokers showed particularly high concentrations of fibronectin and HA (fig. 2) and had measurable PIIIP concentrations in BAL; 1.1, 0.7 and 0.3 $\mu g \cdot l^{-1}$.

The serum FN level in all patients was mean 5.1±1.9 (SD) $mg \cdot l^{-1}$. No significant differences were found between smokers and nonsmokers and no correlation was found between the serum FN and the FN levels in BAL.

Table 1. – The bronchoalveolar lavage (BAL) recovery of fibronectin, hyaluronan and albumin after irradiation therapy of patients with breast cancer, subgrouped for smoking habits

	n	Fibronectin $\mu g \cdot l^{-1}$	Hyaluronan $\mu g \cdot l^{-1}$	Albumin $\mu g \cdot l^{-1}$
All Patients	21	215 (72–640)***	16 (10–27)**	73 (39–135)*
Smokers	11	102 (24–183)	12 (9–17)	58 (38–88)
Nonsmokers	10	489 (199–1202)***	21 (12–37)***	93 (45–193)**
All Controls	19	69 (27–172)	8 (5–16)	47 (31–73)
Smokers	7	73 (24–222)	8 (6–12)	48 (36–66)
Nonsmokers	12	66 (27–159)	10 (5–19)	44 (18–72)

BAL data are represented as geometric mean (±SD). The statistical differences between patients and controls was calculated by using Wilcoxon's non-parametric rank-sum test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

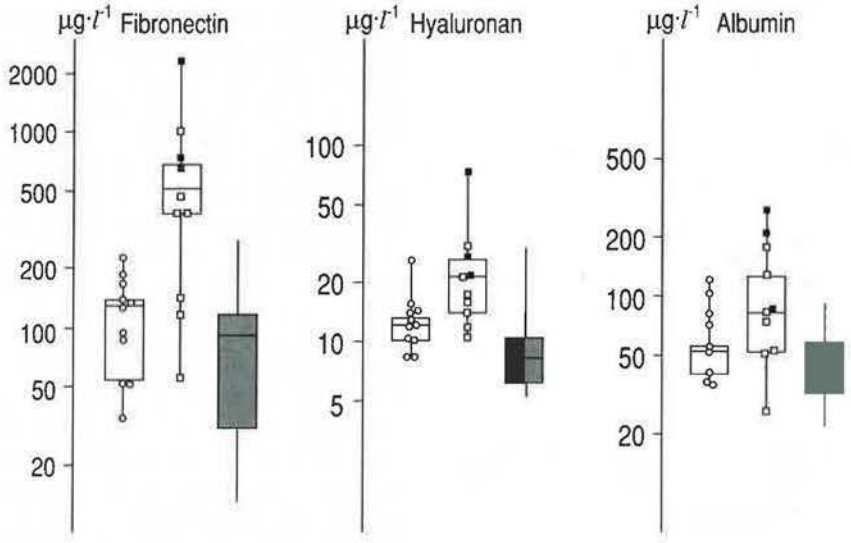

Fig. 2. – The individual BAL concentrations of fibronectin, hyaluronan and albumin in smoking and nonsmoking breast cancer patients (□ and ■) after removal of the tumour and radiation against the operated breast. The box-plots indicate the medians, upper and third quartiles and the ranges. The control group is indicated by shaded box-plots. ○: smokers; □: nonsmoker; ■: nonsmokers who developed pneumonitis on X-ray. BAL: bronchoalveolar lavage.

Table 2. – The cell recovery during bronchoalveolar lavage of breast patients undergoing irradiation therapy

	n	Lymphocytes		Macrophages		Granulocytes		Mast cells	
		$10^7 \cdot l^{-1}$	%	$10^7 \cdot l^{-1}$	%	$10^7 \cdot l^{-1}$	%	$10^7 \cdot l^{-1}$	%
Patients	21	0.5 (0.1–2.6)	9 (2–32)	3.6* (1.8–6.9)	76* (59–98)	0.2 (0.1–0.5)	2.5* (0.9–7.0)	0.01* (0.00–0.07)	0.20*** (0.05–0.77)
Controls	19	0.5 (0.2–1.3)	6 (2–14)	7.9 (3.6–17)	89 (82–98)	0.1 (0.0–0.4)	0.8 (0.2–4.1)	0.00 (0.00–0.02)	0.03 (0.01–0.16)

Data are represented as geometric mean (±SD). The statistical differences between patients and controls was calculated by using Wilcoxon's non-parametric rank-sum test. *: $p<0.05$ compared with the controls.

The cell recoveries are summarized in table 2. The numbers of mast cells were significantly higher in the patient group compared to the controls. A significant increase in the percentage of granulocytes and a decrease in the percentage of, and total numbers of, macrophages compared to the controls was also seen.

In the patient group, a significant correlation was found between BAL fibronectin and the increased total and relative numbers of lymphocytes ($r=0.7$; $p<0.002$ for both correlations). The increase in FN in BAL was also correlated with the increased recovery of HA (fig. 3). A significant correlation was also found between HA and the total ($r=0.49$, $p<0.03$) and relative ($r=0.53$, $p<0.02$) numbers of lymphocytes. There was a tendency towards a correlation between the numbers of mast cells and HA but this was not statistically significant ($r=0.38$, $p<0.1>0.05$). No correlation was found between any of the BAL parameters and the estimated lung volume involved in the radiation field.

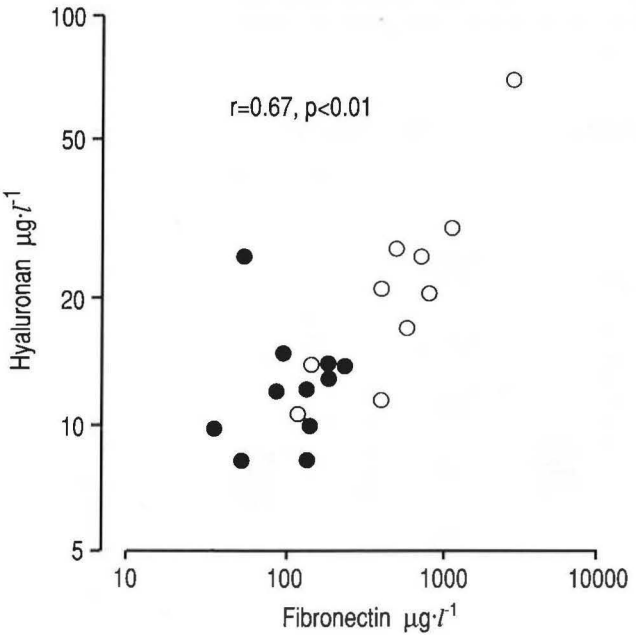

Fig. 3. – The relationship between BAL fluid concentrations of fibronectin and hyaluronan in irradiated patients with breast cancer. Correlation was calculated with Spearman's rank correlation test. ●: smokers; ○: nonsmokers

Discussion

The present study has shown that the post-operative radiation of patients with breast cancer induces, as judged from the BAL findings, a release of fibronectin (FN) and connective tissue components, hyaluronan (HA) and procollagen-III-peptide (PIIIP), from the underlying lung tissue. These signs of an early pulmonary reaction two months after radiotherapy were clearly associated with the smoking habits of the patients.

The mechanisms underlying the radiation-induced alveolar accumulation of fibronectin and HA is not fully understood. Fibronectin is known to be secreted by activated alveolar macrophages, by respiratory epithelium [11, 12] and by fibroblasts [13]. Fibronectin binds to cell surfaces [14, 15] and may contribute to the organization of the extracellular matrix by facilitating cell migration, adhesion and aggregation [16] and by binding to hyaluronan and other glycosaminoglycans [17, 18]. Hyaluronan can be produced by all mesenchymal cells and specifically-activated fibroblasts are prone to synthesize large amounts. In the normal lung, HA is localized in the peribronchial and perivascular tissue [6, 7]. However, after bleomycin or radiation-induced lung damage, large amounts of HA accumulate intra-alveolarly and in the alveolar interstitial tissue [6, 7]. Both FN and HA are important contributors to the early stage of connective tissue response that is seen in radiation-induced fibrosis [10, 19]. Moreover, FN and HA are known to be two major components forming a smooth matrix in the early stage of wound healing [18, 20].

Studies *in vitro* have demonstrated that a number of inflammatory mediators, *e.g.* lymphokines, platelet- and epidermal-derived growth factors are potent stimulators of HA and FN synthesis [13, 21, 22]. In the present study, the increased levels of lymphocytes were related to the increased concentrations of fibronectin and HA. Lymphocytes can secrete mediators that stimulate macrophages to secrete fibronectin [24] and fibroblasts to secrete both FN and HA [23–25]. As outlined in our earlier report [11], we found increased numbers of mast cells in the nonsmoking patient group, whilst no increase was found in the smoking patient group or among the nonsmoking controls. Although in the present study, we could not establish a significant correlation between the increased numbers of mast cells and BAL concentration of HA or FN, we still believe the observation to be important.

In clinical BAL studies on patients with fibrosing interstitial lung disorders, raised levels of FN [26–28] and HA [29, 30] in BAL fluid have been suggested to reflect disease activity. It has previously been reported that the measurement of HA and procollagen peptide in patients with idiopathic pulmonary fibrosis reflects the disease activity and predicts the course of the disease [6] more adequately than cellular analysis. This was also found in an animal experimental study with radiation-induced fibrosis; an increase in HA in contrast to cellular analysis closely paralleled the interstitial accumulation of HA preceding the later formation of fibrosis [31]. The findings in the present study that HA concentrations increased in parallel to FN concentrations suggest that these two factors are involved in the same disease mechanisms.

Procollagen III peptide is another marker of connective tissue turnover and appears in increased concentrations in BAL fluid in interstitial lung diseases with signs of lung fibrosis [32, 33]. The majority of our patients did not have detectable levels of PIIIP in BAL. Interestingly, detectable levels were only seen in four patients, all of who were nonsmokers. Moreover, the three patients with X-ray signs of radiation-induced pneumonitis, all belonged to this group.

One of the main findings in the present study was the observation that the irradiated patients who were smokers displayed no increase in levels of fibronectin and HA in BAL fluid, whilst nonsmoking patients had sevenfold and almost threefold increases in fibronectin and HA, respectively. An inhibitory effect of smoking on the connective tissue response has recently been reported in the bleomycin model in hamsters [34]. Smoking is also reported to be less frequent in patients with extrinsic allergic alveolitis and sarcoidosis [35, 36]. Thus, the present data suggest that smoking may reduce the risk for an interstitial lung reaction. The mechanisms by which smoking reduces a local inflammatory reaction in the lung and effects the connective tissue response can only be speculated upon. Smoking is known to suppress the cell-mediated immune response in man [37] and neutrophil phagocytic activity is also reported to be decreased in smokers [38]. Furthermore, smoking is known to interfere with the antiprotease system favouring degradation of certain connective tissue components (elastin and collagen) and has also been reported to degrade HA [38].

Finally, the present study indicates that smoking interacts with the radiation-induced inflammation and connective tissue response of the lung. We certainly do not recommend nonsmoking patients to take up smoking before radiation treatment. Smoking may, as indicated by the present study, suppress the connective tissue response of the lung. However, smoking may also suppress the therapeutic antitumour effect of irradiation. Such mechanisms have been proposed by others [39].

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