ACTA STEREOL 1994; 13/1: 89-93
PROC 6ECS PRAGUE, 1993
ORIGINAL SCIENTIFIC PAPER

STEREOLOGICAL ANALYSIS BY USE OF CAVALIERI'S PRINCIPLE OF LATE CHANGE IN MOUSE BLADDERS AFTER IRRADIATION AND CHEMOTHERAPY

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ABSTRACT

Stereological analysis by use of Cavalieri's principle of total volume estimation has been performed to investigate the late changes in mouse bladders one year after irradiation alone (10-25 Gy) or irradiation (10-15 Gy) combined with cyclophosphamide (CTX) 100 mg/kg, or cis-diamminedichloroplatinum (II) (cis-DDP), 6 mg/kg. A significant increase in total volume of urothelium was found in the group treated with CTX combined with irradiation. The total volume of connective tissue in the muscular layer in the groups which received irradiation only showed a significant increase. This indicates that irradiation induces development of late damage with connective tissue formation. There also seemed to be an increase in the total amount of connective tissue in muscular tissue, although not statistically significant, in the groups treated with CTX and irradiation compared with CTX alone. This may indicate that CTX prevent the irradiation-induced proliferation of connective tissue. The group treated with CTX and radiation had a significant decrease in the total volume of lamina propria but no difference was found in the other treatment groups when comparing connective tissue, muscular layer or amount of vessels and capillaries with their corresponding control groups. Results from bladders treated with cis-DDP alone or combined with radiation were statistically inconclusive due to the small number of animals in each group. Stereological analysis using Cavalieri's principle of total volume estimation is a feasible method to demonstrate and quantitate treatment-induced late bladder tissue changes.

Key words: bladder irradiation, Cavalieri's principle, chemotherapy, stereological analysis, volume estimation.

INTRODUCTION

Radiotherapy is one of the main options in the treatment of malignant pelvic tumors. Like other modalities used in cancer treatment, radiotherapy can inflict serious damage on the surrounding normal tissues. During the last decade, different chemotherapeutics have been used as adjuvant with the aim of increasing the tumor response however, there is often also an increased risk of normal tissue complications (Withers et al., 1980). Several in vitro animal models have been developed to investigate normal tissue damage after irradiation

alone or combined with chemotherapy (Field and Michalowski, 1979).
Investigations based on histological changes are mainly qualitative or at best semi-quantitative. Semi-quantitative investigations have applied various arbitrary scoring systems; and the main problem remains to obtain quantitative data on the changes in spatial distribution of morphological tissue components which occur following therapy. Cavalieri's direct estimator of volume of objects from sections has soluted this problem (Gundersen et al., 1988).
The aim of the present investigation was to validate this stereological analysis in the histological assessment of late bladder damage after irradiation alone or combined with chemotherapy with special reference to the urothelium, the connective tissue, both in the submucosa and in the muscular layers, and the vessels.

MATERIALS AND METHODS

Animal

Female C_3H/Hen Afnu+ mice were used for the experiments. The mice were all 12-16 weeks old at the start of the experiment, and weighed 25-30 g.

Irradiation and drug treatment

The bladder was irradiated with X-rays generated with a 250 kV constant potential. The irradiation was given as single fractions in a range of doses (10-25 Gy X-rays alone, or 10-15 Gy in combination with chemotherapy). Cyclophosphamide (CTX) and cis-diamminedichloroplatinum (II) (cis-DDP) were given intraperitoneally 45 minutes prior to irradiation at doses of 100 mg/kg and 6 mg/kg, respectively. Control groups of age-matched animals were treated with cis-DDP or CTX alone, or had no treatment.

Stereological analysis

After one year the mice were sacrificed by cervical dislocation. After excision, bladders were left for 24 h in an ethanol:acetic acid:formol:saline fixative (40:5:10:45 v/v) before being transferred to 70% alcohol for storage. They were embedded in paraffin wax before being cut by step sections with a distance of 500 μm from section to section, each section measuring 5 μm in thickness. The first section was taken at a random level between 0 and 500 μm from the top point of the bladder in the paraffin wax. The sections were stained using the Van Gieson method for muscle tissue and collagen.
Every section was magnified and projected onto the table using a Leitz microscope with a 10 x lens and a projection attachment. To estimate the total volume of urothelium, the total volume of connective tissue in the lamina propria and in the muscular layers, the total volume of muscular tissue and the total volume of vessels, Cavalieri's estimator of the volume of arbitrary objects was used (Gundersen et al., 1988). The Italian mathematician (1598-1647) showed that the volume of any object V(obj) may be estimated from parallel sections separated by a known distance t, by summing up the areas of all cross-sections of the object $\Sigma a(obj)$ and multiplying this figure by t:

$$V(obj) = t \cdot \Sigma a(obj) \quad (1)$$

There are no other conditions except that the position of the first section must be random in the object. The Σa(obj) is estimated by applying a test system with regularly arranged points at random on all the projected sections. For each tissue component, all points (P)(tiss.comp.) which hit it are simply counted. The total area of the cross-section of the component is

$$a(\text{tiss.comp.}) = a(\text{point}) \cdot P(\text{tiss.comp.}) \quad (2)$$

where a(point) is the area associated with each point in the test system.

The total volume of each tissue component, V(tiss.comp.), is now estimated after adding points hitting the given tissue component:

$$V(\text{tiss.comp.}) = t \cdot a(\text{point}) \cdot \Sigma P(\text{tiss.comp.}) \quad (3)$$

Statistical methods

All estimated volumes are presented as a group median volume. Comparisons between groups were performed by unpaired Mann Whitney's test.

RESULTS

Table 1 shows the number of bladders examined histologically, the total bladder tissue volume and, and the total median volume of each investigated tissue component.

TABLE 1. Median values of the total bladder tissue volume, urothelium, lamina propria, muscular layer, connective tissue in the muscular layer and vessels

Group	No. of animals	Total bladder volume	Urothelium		Lamina propria		Muscle		Connective tissue/ muscle		Vessels	
		mm^3	mm^3	%	mm^3	%	mm^3	%	mm^3	%	mm^3	%
Control	5	16.6	2.9	17	3.6	22	6.8	40	1.9	11	1.1	7
10 GY	4	15.3	2.8	18	3.3	22	4.5	29	2.8	18	1.0	7
15 GY	15	23.3	3.4	15	3.5	15	12.3	52	3.7	16	0.8	4
20 GY	5	28.0	3.4	12	3.6	13	8.9	32	3.1	11	0.4	1
25 GY	2	4.7	0.4	-	0.3	-	0.4	-	3.4	-	0.1	-
		14.0	1.3	-	4.8	-	4.2	-	3.1	-	0.6	-
CTX	4	17.6	5.4	31	2.3	13	7.0	40	1.9	11	0.9	5
CTX+10GY	4	13.9	3.5	25	2.6	19	4.8	34	3.2	23	1.1	8
CTX+15GY	5	14.6	4.2	29	1.7	12	5.1	35	2.7	18	0.9	6
cis-DDP	3	29.3	3.8	13	6.4	22	14.7	50	3.8	13	1.1	4
cis-DDP	2	17.3	2.3	-	3.3	-	7.6	-	3.1	-	0.7	-
+10 GY		23.9	2.6	-	6.0	-	11.0	-	3.1	-	1.5	-
cis-DDP	2	15.3	2.0	-	2.8	-	6.6	-	3.5	-	0,4	-
+15 GY		12.6	0.6	-	1.5	-	7.6	-	2.8	-	0.1	-

In the groups comprising irradiation alone the total volume of the epithelial tissue was almost similar to that in the control group, but when combined with CTX the increase in epithelial tissue was significant ($p < 0.05$).

The total volume of connective tissue in the muscular layer of the irradiated groups showed a significant increase compared with the control ($p < 0.05$). Increases were also found in the irradiation plus CTX or cis-DDP groups, but these differences were not statistically significant.

The groups comprising CTX± irradiation appeared to show a decrease in the total volume of lamina propria compared with controls but only to a significant degree in the group CTX + 15 Gy ($p < 0.05$). When comparing the remaining treatment groups with the corresponding control groups there was no statistically significant difference in the connective tissue, the muscular layer or the number of vessels.

DISCUSSION

The efficiency of systematic sampling in stereology has recently demonstrated the advantage of describing various tissue components in a strictly quantitative and objective way rather than in the more widespread, subjective or at best semi-quantitative way hitherto employed (Gundersen & Jensen, 1987; Gundersen et al., 1988). The present work was undertaken to apply Cavalieri's method in an experimental long-term study to evaluate changes in tissue components of the bladder wall after irradiation and chemotherapy.

Our study does not reveal an increase in the total volume of urothelium after irradiation alone. This is in contrast to previous cell kinetic studies showing a significantly increased turnover of the urothelium at the time of onset of late damage, represented by thickening of the urothelium similar to the hyperplasia (Stewart, 1986). The increase in total epithelial volume seen after CTX treatment seems to be consistent with previous cell kinetic experiments performed on CBA/Ht mice (Stewart, 1985).

Our results indicate that CTX treatment alone does not cause the development of fibrosis in deeper layers of the bladder. This is consistent with earlier studies performed by Stewart (1985). There seems to be an increase, although not statistically significant in the amount of connective tissue in muscular tissue, evaluated both by total volumes and percentage, in the groups treated with CTX and irradiation compared with CTX alone. This suggests the importance of irradiation in the development of late damage.

A significant increase in the total volume of connective tissue dispersed in the muscular layers in the groups treated with radiation alone was found compared with controls, though there seems to be no relative increase and therefore the difference may merely reflect the increased total bladder volume seen after 15 Gy and 20 Gy. The increased total tissue volume in these groups seems to be especially reflected in the muscular layer. The cause of the increased tissue volume is unclear, but at swelling of the muscular cells before further destruction and cell death (Antonakopoulos et al., 1982) may contribute to the larger volume of these cells.

The results in the groups of animals treated with cis-DDP alone or in combination with irradiation are difficult to interpret since the number of animals is small. The drug itself does not seem to inflict any late damage on the urothelium, in contrast to CTX. This is consistent with recent investigations (Lundbeck & Overgaard, 1992).

Previous reports on histological changes in the bladder after irradiation have been descriptive (Antonakopoulos et al., 1982; Hueper et al., 1942), with no attempt to perform a quantitative estimation of the amount of different tissue components with special reference to late normal bladder tissue damage. To perform quantitative studies of this type Cavalieri's principle of total volume estimation is unbiased efficiency, simple to perform, rapid and unexpensive.

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