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Changes of silver-stained nucleolar organizer regions in mouse endometrial carcinogenesis induced by *N*-methyl-*N*-nitrosourea and 17β-oestradiol

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Summary. A high incidence of endometrial adenocarcinoma and pre-neoplastic lesions was induced in ICR mice treated with *N*-methyl-*N*-nitrosourea and 17β-oestradiol within 23 weeks. The endometrial lesions were histopathologically similar to those of human subjects. To assess the cell proliferative activity of these lesions, a one-step silver colloid staining for nucleolar organizer regions was applied and the numbers of silver-stained nucleolar organizer regions (AgNORs) were counted. The mean numbers ± SD of AgNORs in each lesion were as follows: simple hyperplasia, 2.07 ± 0.36; complex hyperplasia without cytological atypia, 2.79 ± 0.39; complex hyperplasia with cytological atypia, 3.43 ± 0.38; and well-differentiated adenocarcinoma, 4.17 ± 0.40. Significant differences were observed in each lesion ($P < 0.001$). These findings suggest that the mean numbers of AgNORs are increased in the progression of neoplastic changes in the mouse endometrium, as in human endometrial lesions. This rapid induction model of endometrial carcinoma in mice is useful in the understanding of the histogenesis of endometrial carcinoma in human subjects.

Key words: Hyperplasia – Carcinoma – Nucleolar organizer regions – Endometrium – Mouse

Introduction

Nucleolar organizer regions (NORs) are chromosomal segments in which ribosomal RNA (rRNA) is encoded; they contribute to the development of the RNA-containing nucleolus or nucleoli into which the NORs project on large loops of DNA (Underwood and Giri 1988). Recently, NORs have received much attention because of claims that their number per nucleus tends to increase in neoplastic changes in various organs, including the uterine cervix (Rowlands 1988; Yokoyama et al. 1990) and in endometrial lesions (Coumbe et al. 1990; Wilkinson et al. 1990; Niwa et al. 1991 b).

Animal models of neoplasia give us a better understanding of the mechanism of neoplasia but, effective and simple animal models of endometrial carcinoma have not been reported. Recently, we have developed a rapid induction model of endometrial carcinoma in mice treated with *N*-methyl-*N*-nitrosourea (MNU) and 17β-oestradiol (E_2) (Niwa et al. 1991 a). In this model, rapid induction (within 23 weeks) with a high incidence of endometrial neoplastic and pre-neoplastic lesions was observed. In the present study, proliferative potential of pre-neoplastic and neoplastic lesions of endometrium induced by MNU and E_2 in mice was evaluated by measuring silver-stained NORs (AgNORs) with the one-step colloid method devised by Howell and Black (1980).

Materials and methods

A total of 46 female ICR mice, 10 weeks of age, were purchased from Charles River Japan (Kanagawa, Japan) and divided into two experimental groups and treated as shown in Fig. 1. Group 1 (31 mice) was given three intravaginal instillations of MNU (purchased from Nacalai Tesque, Kyoto, Japan) using a metal syringe at a dose of 1 mg/100 g body weight once a week for 3 weeks, and then fed the diet containing 5 ppm E_2 for 20 weeks, starting 1 week after the last exposure of MNU. Group 2 (15 mice) was fed the basal diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and served as an untreated control. The experiment was terminated

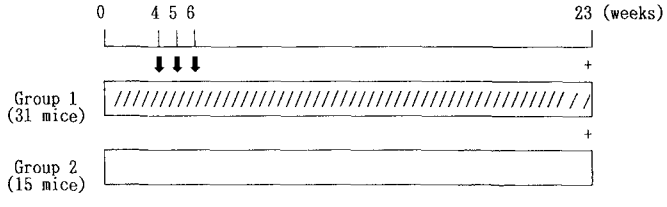

Fig. 1. Experimental protocol. ⬇, Intravaginal instillation of MNU (1 mg/100 g body weight); +, sacrifice; ▨, 5 ppm E_2 containing diet; ▭, basal diet (oriental MF)

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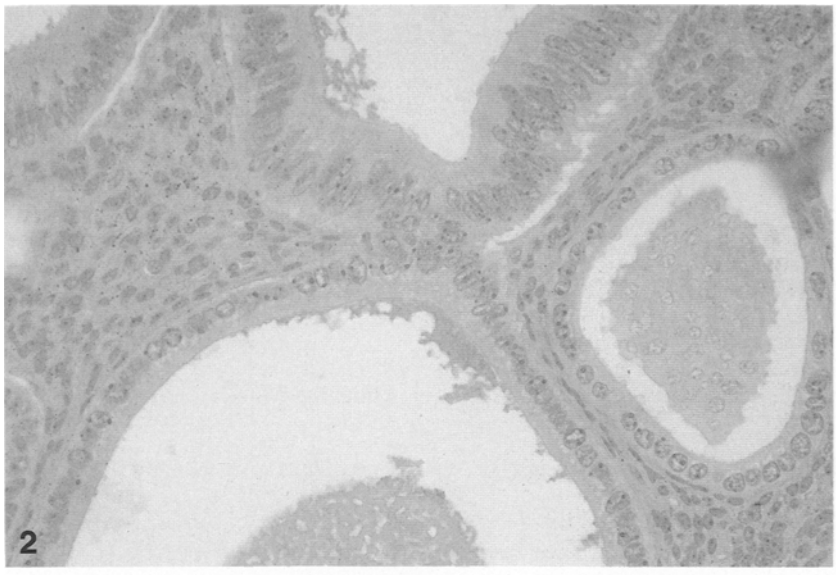

Fig. 2. Simple hyperplasia of the endometrium in group 1. One or two silver dots are present in each nucleus. Silver colloid stain, ×340

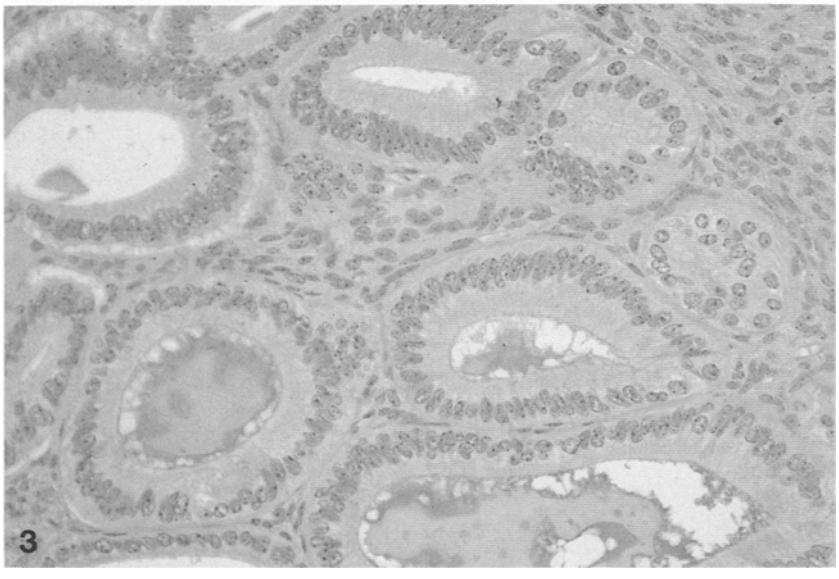

Fig. 3. Complex hyperplasia without cytological atypia of the endometrium in group 1. Two or three silver dots are found in each nucleus. Silver colloid stain, ×340

Table 1. Incidences of pre-neoplastic and neoplastic lesions in each group

Experimental groups	Number of animals initiated	Effective number of animals[a]	Number of animals with			
			Simple hyp	Com hyp		Adeno-carcinoma
				without atypia	with atypia	
Group 1 (MNU + E_2)	31	31	31 (100%)	22 (71%)	17 (55%)	15 (48%)
Group 2 (no treatment)	15	13	0	0	0	0

[a] Animals survived more than 10 weeks
Simple hyp, Simple hyperplasia; Com hyp, complex hyperplasia

23 weeks after the start. At the termination of the experiment, all surviving animals were killed and autopsied. The uterus and ovaries were taken for histological examination. Tissues were processed for histology by the conventional method, and two serial sections (3 μm in thickness) were prepared. One was stained with haematoxylin and eosin for diagnosis and the other was stained for AgNORs.

Uterine endometrial lesions were basically diagnosed according to Scully's criteria (1982) by more than two pathologists and were divided into four lesions: simple hyperplasia, complex hyperplasia

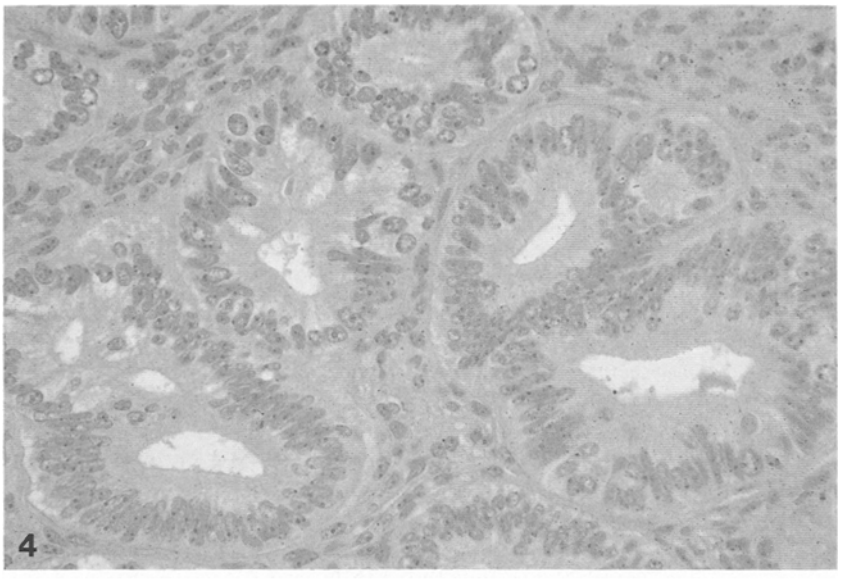

Fig. 4. Complex hyperplasia with cytological atypia of the endometrium in group 1. Three or four silver dots are present in each nucleus. Silver colloid stain, ×340

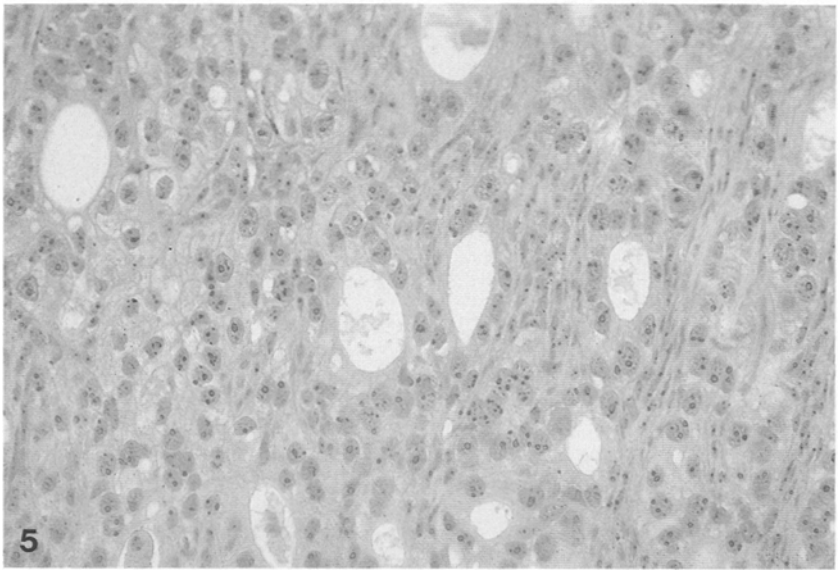

Fig. 5. Well-differentiated adenocarcinoma of the endometrium in group 1. Four or five silver dots are present in each nucleus. Silver colloid stain, ×340

without cytological atypia, complex hyperplasia with cytological atypia, and adenocarcinoma.

AgNOR staining was performed as reported by Howell and Black (1980): briefly, sections were dewaxed in xylene and then hydrated through ethanol to distilled water. A solution of gelatine at a concentration of 2 g/dl in aqueous formic acid was mixed, 1:2 volumes, with 50% aqueous silver nitrate. After filtration with Millipore (0.22 μm), the filtrate was dropped onto the sections, which were left in the dark at room temperature. The reaction was stopped after confirming nucleolar staining under the microscope. The reaction time was between 30 and 60 min. The sections were then washed with distilled water, and mounted in a synthetic medium.

To count the AgNORs sections were examined under a ×100 oil immersion lens. In all the specimens, the numbers of AgNOR dots in more than 100 randomly selected cells of each lesion were counted by three cytopathologists (K.N., Y.Y. and T.T.). Small (pre)neoplastic lesions, consisting of under 100 cells, were omitted from the present study. Nucleolar aggregates of AgNORs were counted as a single AgNOR dot.

The results were analysed by Student's *t*-test.

Results

In group 1, a high incidence of endometrial pre-neoplastic and neoplastic lesions in the uterine corpus was observed within 23 weeks (Table 1). Of these lesions, 30 cases of simple hyperplasia, 22 of complex hyperplasia without cytological atypia, 10 with complex hyperplasia with cytological atypia, and 11 adenocarcinomas were found and used for counting AgNORs. In group 2, 8 proliferative phase and 3 secretory phase endometria were studied for AgNOR staining. AgNORs were clearly recognized as black dots in cell nuclei (Figs. 2–5). The mean numbers ± SD of AgNORs in the various lesions are summarized in Table 2, and were as follows: simplex hyperplasia, 2.07 ± 0.36; complex hyperplasia without cytological atypia, 2.79 ± 0.39; complex hyperplasia with cytological atypia, 3.43 ± 0.38; and adenocarcinoma, 4.17 ± 0.40. Significant differences were found in each

Table 2. Mean number of silver-stained nucleolar organizer regions (AgNORs) per nucleus in each endometrial lesions

Endometrium	Number of specimens examined	Number of AgNORs
Glandular cells in the proliferative phase	8	1.98±0.38
Glandular cells in the secretory phase	3	1.77±0.14
Simple hyperplasia	30	2.07±0.36[a]
Complex hyperplasia without cytological atypia	22	2.79±0.39[b]
Complex hyperplasia with cytological atypia	10	3.43±0.38[b]
Adenocarcinoma	11	4.17±0.40[b]

[a] Mean±SD
[b] Significant differences were present between each lesion ($P<0.001$)

lesion ($P<0.001$). As for normal endometrium, proliferative and secretory phase endometrium were 1.98 and 1.77, respectively. No significant differences were observed between these two phases of the endometrium.

Discussion

The results of this study show that the mean numbers of AgNORs in mouse endometrial pre-neoplastic and neoplastic lesions tended to increase with the progression of neoplastic changes. Significant differences are present between each pre-neoplastic and neoplastic lesion.

In human endometrial carcinomas, two different pathogeneses for the neoplasms have been considered (Bokhman 1983; Deligdisch and Holinka 1987). One is associated with pre-neoplastic lesions, such as complex hyperplasia without cytological atypia, and the other is not (Deligdisch and Holinka 1987). The former is considered to be associated with abnormal hormonal profiles and as with exogenous oestrogen administration (Deligdisch and Holinka 1987), and sometimes affects younger women. If it is necessary to preserve the uterus for child-bearing, hormonal therapy may be effective and the results of the present study suggest that assessing the AgNORs may be a possible method for distinguishing the endometrial pre-neoplastic and neoplastic lesions in this group.

The number of AgNORs is thought to be one method of assessing cellular proliferation (Hall and Levison 1990; Quinn and Wright 1990) and has also been shown to be a diagnostic significance between neoplastic and preneoplastic lesions, including those of the human liver (Crocker and McGovern 1988), uterine cervix (Rowlands 1988), uterine endometrium (Coumbe et al. 1990; Wilkinson et al. 1990; Niwa et al. 1991b) or rat liver (Tanaka et al. 1989). The number of AgNORs correlates with that of cells labelled with monoclonal antibody Ki-67, which is considered to be a marker of cell proliferation (Hall et al. 1988). In experimental rat liver carcinogenesis, the mean number of AgNORs has also been reported to correlate with bromodeoxyuridine-labelling index, which reflects cell proliferation (Tanaka et al. 1989). In this study, the mean numbers of AgNORs were increased in the neoplastic changes and are significantly different between each pre-neoplastic and neoplastic lesion. These results are consistent with those of our previous studies using human endometrial lesions (Niwa et al. 1991b).

Members of the *ras* gene family are thought to be most frequent oncogenes found in human tumours (Balmain and Brown 1988; Barbacid 1987) and in endometrial carcinoma, a relatively high incidence of point mutation in codon 12 of K-*ras* oncogene was reported after detection by the polymerase chain reaction (Enomoto et al. 1990; Lester and Cauchi 1990). In mouse mammary carcinogenesis induced by MNU, point mutation in codon 12 of K-*ras* was also detected (Miyamoto et al. 1990). Point mutation in codon 12 of K-*ras* in the present model induced by MNU and E_2 is possible and studies for the detection of this point mutation are in progress. The endometrial pre-neoplastic and neoplastic lesions of the model are similar to those of humans histopathologically, especially adenocarcinoma with hyperoestrogenic conditions (Niwa et al. 1991b). If the point mutation is detected, our model is very like human endometrial adenocarcinoma.

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