ORIGINAL PAPER

Clonal evolution and progression of 20-methylcholanthrene-induced squamous cell carcinoma of mouse epidermis as revealed by DNA instability and other malignancy markers

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SUMMARY

We examined the clonal evolution of skin malignant lesions by repeated topical applications of 20-methylcholanthrene (20-MC) to the skin, which induces hyperplastic epidermis, papillomatous lesion and invasive carcinoma in mice. The lesions were examined histologically and immunohistochemically with anti-single-stranded DNA after acid hydrolysis (DNA-instability test), p53, VEGF, DFF45, PCNA and AgNORs parameters analyses. Multiple clones with increased DNA instability comparable to that of invasive carcinoma were noted in early-stage (2-6 weeks) hyperplastic epidermis, and their number increased in middle (7-11 weeks), and late-stages (12-25 weeks) of hyperplastic epidermis, indicating that they belong to the malignancy category. All papillomatous lesions and invasive carcinomas showed a positive DNA-instability test. Positive immunostaining for various biomarkers and AgNORs parameters appeared in clones with a positive DNA-instability test in early- or middle-stage hyperplastic epidermis, and markedly increased in late-stage hyperplastic epidermis, papillomatous lesions and invasive carcinomas. The percentage of PCNA-positive vascular endothelial cells was significantly higher in VEGF-positive lesions with a positive DNA-instability test and became higher toward the late-stage of progression. Cut-woundings were made to papillomatous and invasive carcinoma lesions, and the regeneration activity of vascular endothelial cells was determined by using flash labeling with tritiated thymidine ($_3$H-TdR). In small papillomatous lesions, vascular endothelial cells showed regenerative response, but the response was weak in large lesions. No such response was noted in invasive carcinomas; rather, cut-wounding induced collapse of blood vessels, which in turn induced massive coagulative necrosis of cancer cells. These responses can be interpreted to reflect exhausted vascular growth activity due to excessive stimulation by VEGF-overexpression, which was persistently seen from hyperplastic epidermis to invasive carcinoma.

INTRODUCTION

Several groups have investigated the immediate and long-term effects of single or repeated topical surface applications to the mouse dorsal skin of various chemical carcinogens (Iversen and Ever-

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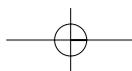
sen, 1962; Iversen and Bjerkbes, 1963; Iversen *et al.*, 1976; Fukuda *et al.*, 1978; Iversen *et al.*, 1982; Colapietro *et al.*, 1993; Ishikawa *et al.*, 1999; Reddig *et al.*, 1999; Owen *et al.*, 1999; Saran *et al.*, 1999; Wei *et al.*, 1999; Coghlan *et al.*, 2000). These studies concluded that chemical carcinogens could cause a variety of skin lesions ranging from hyperplastic epidermis to invasive carcinoma.

Recent advances in molecular biology have allowed the detection of changes in various biomarkers in the carcinogen-treated mouse epidermis during the course of carcinogenesis. For example, activated Ha-ras oncogene with carcinogen-specific point mutations has been demonstrated in the mouse skin epidermis treated with various chemical carcinogens at various stages of carcinogenesis (Brown *et al.*, 1990; Bowden *et al.*, 1991; Nelson *et al.*, 1992; Vassar *et al.*, 1992; Kemp *et al.*, 1993, 1994; Finch *et al.*, 1996; Larcher *et al.*, 1996; Tober *et al.*, 1998). Furthermore, cyclin D1 gene rearrangement, amplification and protein overexpression, indicating cyclin deregulation, have been found in mouse skin papillomatous lesions induced by carcinogens (Robles *et al.*, 1995; Stern *et al.*, 1998). Similarly, overexpression of transforming growth factor (TGF) α (Vassar *et al.*, 1992) or β (Cui *et al.*, 1994; Stern *et al.*, 1998) was demonstrated to play a central role in chemically-induced papilloma formation and carcinogenesis (Go *et al.*, 1999). Changes in the expression of keratins have been reported during the course of chemically-induced mouse skin carcinogenesis (Gimenz-Conti *et al.*, 1990; Huitfeldt *et al.*, 1991; Caulin *et al.*, 1993; Stern *et al.*, 1998). Moreover, mutations in the p53 gene were identified in the late stages of chemically-induced tumor progression and were closely associated with poorly differentiated phenotype (Ruggeri *et al.*, 1991) and the lack of such phenotype in p53 knockout mice greatly enhanced malignant progression (Kemp *et al.*, 1993) in mouse skin chemical carcinogenesis. Immunohistochemically identified cells positive for mutant p53 protein showed close intercorrelation with the degree of histological dysplasia induced by chemical carcinogens (Lu *et al.*, 1997; Stenback *et al.*, 1998).

Angiogenesis is a crucial process for tumor growth, and vascular endothelial growth factor (VEGF) is a specific mitogen for endothelial cells (Leung *et al.*, 1989), which is overexpressed at the later stages of mouse skin chemical tumor progression (Larcher *et al.*, 1996;

Bolontrade *et al.*, 1998; Tober *et al.*, 1998).

DNA fragmentation factor (DFF) is an important factor in the cascade leading to apoptosis. DFF is activated by caspase-3 and is involved in the formation of nuclear DNA fragments. DFF is a heterodimeric protein of 40 kDa and 45 kDa, which is activated when it is cleaved by caspase-3. Of the two enzymatically cleaved fragments, the 40 kDa fragment (DFF40) is the active component responsible for triggering chromatin condensation (Liu *et al.*, 1997; Enari *et al.*, 1998; Sabol *et al.*, 1998; Sakahira *et al.*, 1998; Sasaki *et al.*, 1999).

Cell proliferation as well as apoptosis-related factors such as p53 and DFF are useful features to allow investigating the dysfunctional proliferative regulation of cancerous transformation. The proliferative activity in chemically-induced lesions has been evaluated by immunohistochemical staining of different cell proliferation-related proteins such as MIB-1 (Soballe *et al.*, 1996), PCNA (Cui *et al.*, 1994; Stenback *et al.*, 1998; Stern *et al.*, 1998; Zhang *et al.*, 1998) and AgNORs (Carbonelli *et al.*, 1994). In all the above studies, significant increases were detected in cellular proliferation during various phases of neoplastic transformation.

The above studies also detected abnormal gene expression and protein synthesis, disturbed cytodifferentiation, and stimulated proliferative activity during the course of progression of malignancy, but the results were not consistent and no specific markers were identified for detecting malignant clones, although the abnormalities were statistically significant.

Fukuda *et al.* (1986, 1993) found that nuclear DNA of malignant neoplastic cells is always and without exception much more unstable than that of comparable benign tumor cells and normal cells, irrespective of epithelial or mesenchymal origin, thus yielding a larger amount of single-stranded DNA by acid hydrolysis (2N HCl, 30°C for 20 min). They developed a method for differential fluorescent or immunohistochemical staining of malignant neoplastic cells after denaturation of nuclear DNA by acid hydrolysis. They further used the technique to identify malignant clones in so-called border-line neoplastic lesions (Nitta *et al.*, 1993; Otaki *et al.*, 1994; Tsuzuki *et al.*, 1994; Azuchi *et al.*, 1998; Khaled *et al.*, 2000).

In the present study, we used the above method to detect malignant clones by increased DNA instability (DNA-instability test) during the course of mouse skin hyperplastic epidermis, papillomatous

lesion and invasive carcinoma induced by repeated topical applications of 20-methylcholanthrene (20-MC). In order to identify the biological characteristics of these malignant clones of positive DNA-instability test, immunostaining for other biomarkers such as p53, VEGF, DFF45, PCNA, and AgNORs was also examined. Double immunostaining for CD34 and PCNA was also applied to the lesions to demonstrate the direct intercorrelation between VEGF production and proliferative activity of vascular endothelial cells in the neighboring stromal tissue. In order to examine the reactive changes of vascular endothelial cell proliferation in response to wound stimulation, cut woundings were made in papillomatous lesions and invasive carcinomas and were labelled with tritiated thymidine ($_3$H-TdR) for autoradiography.

MATERIALS AND METHODS

Animals and carcinogen treatment

Male mice (dd-strain, 5 weeks old, Japan SLC Co., Tokyo, Japan) were used in all experiments. They were housed in plastic cages in a barrier-sustained animal room with a 12/12 h light/dark cycle at 22±2°C and 55±5% relative humidity, and provided with commercial pellet diet (Clea Japan Inc., Tokyo) and water *ad libitum*. After 1-week acclimation, the dorsal region of each mouse was clipped clean of hair with an electric hair clipper. 20-MC solution (0.5%, Sigma-Aldrich, St. Louis, MO, USA) dissolved in acetone was applied topically twice a week on the dorsal epidermis. A total of 100 mice were used for the present study. Mice were sacrificed by cervical dislocation under ether anesthesia. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Fukui Medical University.

Immunohistochemical staining of paraffin-embedded sections with polyclonal anti-single-stranded DNA antibody after acid hydrolysis to demonstrate DNA instability (DNA-instability test)

A total of 70 mice with hyperplastic epidermis, papillomatous lesions and invasive carcinoma (2-25 weeks) were used for these studies. All materials were fixed with buffered 10% formalin for 24 h at room temperature, and 4 µm paraffin embedded sections were prepared. They were deparaffinized and rehydrated by ethanol.

(a) Preblocking

After washing in water, intrinsic peroxidase was blocked with 0.03% H_2O_2 solution dissolved in absolute methanol at 20°C for 15 minutes and rinsed with PBS (pH 7.4). The sections were mounted with 2% skim-milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 20 min to block the background adsorption of antiserum. Then they were reacted with the blocking solution (Histofine, SAB-PO, Nichirei, Tokyo) at 20°C for 5 min.

(b) HCl hydrolysis and immunohistochemical staining of single-stranded DNA by the ABC method

Sections were then washed in water, followed by acid hydrolysis with 2N HCl at 30°C for 20 min, and then washed in water again. They were reacted overnight with polyclonal antibody to cytidine (Biogenesis, ONF, UK, 1:1,500 dilution with PBS, pH 7.4) at 4°C then rinsed with PBS (pH 7.4). Sections were reacted with biotinylated goat anti-rabbit IgG (Histofine) at 37°C for 40 min, rinsed with PBS (pH 7.4) and allowed to react with the avidin-biotin peroxidase complex (Histofine) at 37°C for 30 min, then rinsed with PBS (pH 7.4).

(c) Co-DAB reaction

In order to visualize the peroxidase color reaction, sections were incubated with DAB (3,3'-diaminobenzidine tetrahydrochloride) cobalt solution (DAB, Dogin, Kumamoto, Japan, 50 mg dissolved in 100 ml of 0.05M Tris-HCl buffer, pH 7.4, added with 2 ml of 1% $CoCl_2$ and 10 µl of 30% H_2O_2 solution) at 20°C for 10 min and washed in water. Nuclear counterstaining was carried out with Kernechtrot (Kernechtrot, Chroma, Stuttgart, Germany, 100 mg and 5 g aluminum sulfate dissolved in 100 ml distilled water).

Immunohistochemical staining of p53, VEGF, DFF45 and PCNA

Serial 4 µm thick sections were prepared from the same specimens used for the DNA-instability test as described above. After washing in water, intrinsic peroxidase was blocked with 0.03% H_2O_2 solution dissolved in absolute methanol at 20°C for 15 min and rinsed with PBS (pH 7.4). Sections were irradiated in a poly-propylene slide holder with a cap filled with 10 mM Na-citrate buffer (pH 6.0), over a period of 10 min, using an autoclave (Hirayama, 1.5kW, Tokyo, Japan), then mounted with 2% skim-milk (Yukijirushi) dissolved in PBS (pH 7.4), at

37°C for 30 min to block the background adsorption of antiserum. They were further reacted with the blocking solution (Histofine) at 20°C for 5 min. This was followed by reactions with the following primary antibodies, respectively, at 4°C overnight; monoclonal anti-p53 antibody (Ab-3, Calbiochem, MA, USA, 1:100 dilution with PBS, pH7.4), polyclonal anti-VEGF antibody (Neomarkers, CA, USA, 1:100 dilution with PBS pH 7.4), polyclonal anti-DFF45 antibody (Novocastra, Newcastle, UK, 1:200 dilution with PBS, pH 7.4), monoclonal anti-PCNA antibody (PC-10, Calbiochem, 1:100 dilution with PBS, pH 7.4). Then the sections were rinsed with PBS (pH 7.4) and further reacted with biotinylated anti-mouse IgG (Histofine) for monoclonal anti-p53 and anti-PCNA antibodies, and with biotinylated anti-rabbit IgG (Histofine) for polyclonal anti-VEGF and anti-DFF45 antibodies, respectively, followed by rinsing with PBS (pH 7.4). They were allowed to react with the avidin-biotin-peroxidase complex (Histofine) at 37°C for 30 min and rinsed with PBS (pH 7.4). In order to visualize the peroxidase color reaction, sections were incubated with DAB solution (10 mg dissolved in 50 ml PBS, pH 7.4) at 20°C for 10 min and washed in water. Nuclear counterstaining was performed with hematoxylin.

Evaluations of immunohistochemical staining for DNA-instability test, p53, VEGF, DFF45 and PCNA

As for the DNA-instability test, foci with more than several positively stained epithelial cells were regarded as positive. The PCNA-index of hyperplastic epidermis, papillomatous lesions and invasive carcinoma represented the percentage of positively stained cells among more than 500 epithelial cells in each lesion, and was determined in both DNA-instability test-positive and -negative areas, respectively. In the same areas in serial sections, foci with >5% cells positively stained for p53, VEGF and DFF45 were regarded positive after counting more than 500 epithelial cells.

AgNORs

Specimens taken from the same tissues used in the above experiments were used for AgNORs assay. Deparaffinized and ethanol rehydrated specimens were washed in de-ionized and distilled water, and coated with 2 g/dl gelatin dissolved in 1 mg/dl formic acid solution added with twice the volume of 50 g/dl silver nitrate, and allowed to react at 20°C for 15 min under a safety light (No. LA. Kodak, NY).

The specimens were then fixed with Fuji-fix solution (Fujifix, Fuji Photo Film, Tokyo, 445 g dissolved in 2,000 ml of de-ionized and distilled water) for 1 min at room temperature and thoroughly washed in de-ionized and distilled water. No nuclear counterstaining was performed.

Quantitative analysis of the number, mean and largest sizes and maximum shape irregularity of AgNORs

The color image analysis software MacScope ver 2.56 (Mitani Corp., Fukui, Japan) was used. The image of an optical field of a specimen stained by the AgNORs technique (viewed by x1000 objective) was projected on the cathode ray tube (CRT). The total number of AgNORs in one optical field or in each nucleus was counted manually. The total area (μm^2) of all AgNORs-positive nuclei in one optical field or in each nucleus was automatically determined by the image analyzer software. The mean ± SD of AgNORs area in each optical field or in each nucleus was computed. Three to five optical fields, i.e., about 100 cells were examined in each specimen. The mean value of the maximum shape irregularity of AgNORs expressed as the degree of deviation from a regular circle, was computed for the most irregularly shaped AgNOR in 10 nuclei in each specimen, by the same application, using a printed photograph of the image viewed on the CRT with a final magnification of x 1,000.

Immunohistochemical double staining for CD34 and PCNA

Serial 4 µm thick sections prepared from the same specimens used for other staining procedures were used in these assays. After washing in water, the intrinsic peroxidase was blocked with 0.03% H_2O_2 solution dissolved in absolute methanol at 20°C for 15 min and rinsed with PBS (pH 7.4). Sections were irradiated in a poly-propylene slide holder with a cap filled with 10 mM Na-citrate buffer (pH 6.0), over a period of 10 min, using an autoclave (Hirayama, 1.5 kW, Tokyo), then mounted with 2% skim-milk dissolved in PBS (pH 7.4), at 37°C for 30 min to block the background adsorption of antiserum. They were further reacted with the Histofine blocking solution at 20°C for 5 min, followed by reaction with monoclonal rat anti-mouse CD34 antibody (CBR-E8, Southern Biotechnology Associates, Birmingham, AL, USA, 1:200 dilution with PBS, pH 7.4) at 4°C overnight. They were then rinsed with PBS (pH 7.4) and further reacted with

biotinylated goat anti-rat IgM (A104BS, American Qualex International, CA, USA, 1:50 dilution with PBS, pH 7.4) at 37°C for 40 min, followed by rinsing with PBS (pH 7.4). Sections were allowed to react with Histofine avidin-biotin-peroxidase complex at 37°C for 30 min and rinsed with PBS (pH 7.4). In order to visualize the peroxidase color reaction, sections were incubated with DAB solution (10 mg dissolved in 50 ml PBS, pH 7.4) at 20°C for 10 min and washed in water. Then sections were reacted with monoclonal anti-PCNA antibody (pH 7.4) at 4°C overnight, rinsed with PBS, pH 7.4, and further reacted with biotinylated anti- mouse IgG at 37°C for 40 min, followed by rinsing with PBS (pH 7.4). They were allowed to react with the avidin-biotin peroxidase complex at 37°C for 30 min, then rinsed with PBS. For the peroxidase color reaction, sections were incubated with DAB cobalt solution at 20°C for 10 min and washed in water. Nuclear counterstaining was carried out with Kernechtrot.

Quantitative analysis of immunohistochemical double staining for CD34 and PCNA

The percentage of PCNA-positive capillary endothelial cells (PCNA-index) in the vicinity of epithelial cell clones positive for VEGF was determined by counting at least 100 endothelial cells. The percentages of VEGF negative zones were also determined as the control.

Wounding experiments

(a) Procedure

A total of 30 mice were used in these experiments. A linear wound was made under ether anesthesia using a razor blade. Papillomatous lesions and invasive carcinomas were incised to the muscle layer.

(b) Flash or instantaneous DNA-labeling with $_3$H-TdR

Flash DNA-labeling was performed by sacrificing animals 30 min after a single intraperitoneal injection of tritiated thymidine ($_3$H-TdR, approximately 2.0 mCi/g, specific activity, 23 Ci/mM, Radioisotope Center, Tokyo) by using 5 animals each before and 24 h after cut-wounding of small (0.5 cm in diameter), middle (1.0 cm in diameter) and large (1.5 cm in diameter) papillomatous lesions, and before and 10 and 20 days after cut wounding for invasive carcinoma lesions. All DNA-labeled materials were fixed in 10% buffered formalin for 24h at room temperature and

4 μm paraffin-embedded sections were prepared and deparaffinized and ethanol replaced as described above.

(c) Autoradiography

All sections were dipped in melted nuclear emulsion (NR-M2, Sakura, Kyoto, Japan) and autoradiographs were developed in FD 111 at 24°C for 8 min after exposure in a refrigerator for 4 weeks. The autoradiographs were stained with hematoxylin and eosin.

(d) Quantitative analysis of autoradiography

The percent labeling index of capillary endothelial cells was determined for small, middle and large papillomatous lesions in three different areas, top, middle and basal zone of a capillary. More than 200 endothelial cells were counted to determine the labeling index of each capillary zone in both papillomatous lesions and invasive carcinoma lesions.

Statistical Analysis

PCNA-indices (epidermal cell and vascular endothelial cell) and AgNORs parameters are presented as mean ± SD. Data were analyzed using Students t-test with a *p* value less than 0.05 considered significant. Comparison of positive lesions for p53, VEGF, DFF45 and DNA-instability test was performed by the chi-square test, the selected level of significance was *p*<0.05. Comparison of different biological parameters (PCNA-indices and AgNORs parameters) in DNA-instability test-positive and negative cases were analyzed using Students t-test with a *p* value less than 0.05 considered significant.

RESULTS

The histological features of hyperplastic epidermis (2-25 weeks after exposure to 20-MC), papillomatous lesion (7-25 weeks), and invasive carcinoma (12-25 weeks) were determined by routine HE staining. Hyperplastic epidermis were subdivided into three stages, early- (2-6 weeks, before the onset of papillomatous lesions), middle- (7-11 weeks, the hyperplastic epidermis remaining between papillomatous lesions) and late- (12-25 weeks, marginal hyperplastic epidermis continuous with the papillomatous lesions or invasive carcinoma lesions) stages. The normal epidermis of mouse dorsal skin

is composed of 2-3 layers of squamous cells but the hyperplastic epidermis induced by carcinogen treatment at the early- (3-5 layers), middle- (5-10 layers) and late- (more than 10 layers) stages showed thickening with increased cell layers. However, the degree of cellular atypia in hyperplastic epidermis was mild, and no morphological sign of malignancy was noted. Papillomatous lesion cells had slightly enlarged nuclei with enlarged nucleoli but the polarities were still preserved without invasion and were regarded as benign morphologically. In invasive carcinomas, the loss of polarity with invasion and increased degree of cellular atypia were obvious. Clones with histological characteristics similar to invasive carcinoma but without invasion were also present in the papillomatous lesions.

Immunohistochemical staining

The percentages of cells positive for p53, VEGF, DFF45, PCNA and AgNORs parameters in relation to the DNA-instability test are summarized for the three types of lesions in Table I.

(a) DNA-instability test

After hydrolysis with 2N HCl at 30°C for 20 min, all cancer cells in all cancer lesions showing invasion (20 lesions) were positively stained without exception with anti-single-stranded DNA antibody indicating increased DNA instability (Fig. 1c). While normal epidermal cells, normal vascular endothelial cells and fibroblasts present in the same slides were all negative.

In 15 sections (1.5 cm long) of early-stage hyperplastic epidermis, only 2 (13.3%) small foci of positively stained cells were detected, but the number increased in the middle (7 foci/15 sections, 46.7%) and late (31 foci/40 sections, 77.5%) stages (Fig. 1a). In hyperplastic epidermis, DNA instability positive cells were distributed in a few basal-side layers in the early-stage and almost throughout the entire thickness of the epidermis in the middle to late-stages. Even in the late-stages of hyperplastic epidermis, however, foci with a negative DNA-instability test were still seen between the positively stained foci. However, all cells in all papillomatous lesions (30 lesions) were diffusely positive by the DNA-instability test (Fig. 1b).

(b) p53 immunohistochemistry

Normal epidermis was negative for p53 staining. Epidermal cells positive for p53 were noted in hyperplastic epidermis and their distribution coin-

cided well with the foci of positive DNA instability, at the middle (1 foci/7 foci of positive DNA instability, 14.3%) and late-stages (6 foci/31 foci of positive DNA instability, 19.4%), but the number of positive foci was rather small (Fig. 1d). No positive foci were found in 2 foci of positive DNA instability in the early-stage of hyperplastic epidermis. In hyperplastic epidermis, cells positively stained for p53 were distributed in 3-4 layers of the basal side of the epidermis.

In papillomatous lesions, the number of positively stained cells increased and the cells in the lower 5-6 layers were all diffusely positive for p53 (Fig. 1e). Among a total of 30 papillomatous lesions that were all positively stained in the DNA instability test, 14 (46.7%) lesions showed positive staining for p53 and p53-positive cells were often distributed throughout the entire thickness of the lesions. In 20 invasive carcinoma lesions with positive DNA instability, 13 (65%) showed positive p53 staining, which was noticed to be diffusely distributed throughout the entire lesion (Fig. 1f). The percentages of middle and late-stage hyperplastic epidermis with positive DNA instability, papillomatous lesions and invasive carcinoma lesions that were positive for p53 were significantly higher than normal and hyperplastic epidermis with negative DNA-instability (p<0.05, each, Table I).

(c) VEGF immunohistochemistry

Immunohistochemical staining for VEGF showed homogeneous cytoplasmic staining. The distribution of VEGF-positive cells coincided with the foci of positive DNA instability. The percentages of VEGF-positive cells among foci with positive DNA instability were 0, 28.6, 41.9% in early, middle and late-stage hyperplastic epidermis lesions (Fig. 1g), respectively, while those of papillomatous lesions and invasive carcinoma were 60 and 80%, respectively. The differences between groups were significant (p<0.05) (Fig. 1h, i, Table I). Normal epidermis and hyperplastic epidermis with a negative DNA-instability test were all negative for VEGF.

(d) DFF45 immunohistochemistry

Immunohistochemical staining for DFF45 showed granular cytoplasmic and perinuclear staining patterns. Normal epidermis and hyperplastic epidermis with a negative DNA-instability test were all negative. The distribution of DFF45-positive cells coin-

Table I

The results of immunohistochemical staining for p53, VEGF, DFF45, PCNA, and double staining for CD34 and PCNA, and the quantitative analyses of AgNORs parameters. Statistical analyses by Students t-test were carried out for the values of PCNA-indices and AgNORs parameters using the values for normal cases as the standard. The values with meaningful differences (p<0.05) are shown by single stars. Comparison of the positivities of p53, VEGF, DFF45 and DNA-instability test between two different lesions was made by the Chi-square test. The cases with meaningful differences are shown by double stars (p<0.05). Comparison of the values of PCNA-index and AgNORs parameters in the cases of positive and negative DNA-instability tests was made by Students t-test, and the values with meaningful differences are indicated by triple stars (p<0.05).

DNA-instability test	p53 (%)	VEGF (%)	DFF45 (%)	PCNA (epidermal cell) (%) (mean±S.D.)	double staining for CD34 and PCNA (endothelial cell) (%) (mean±S.D.)	number (mean±S.D.)	AgNOR mean area (μm²) (mean±S.D.)	mean total area in one number (mean±S.D.)	largest size (μm²) (mean±S.D.)	maximum shape irregularity (mean±S.D.)
Normal epidermis										
all-negative (n=10)	0 (0/10)	0 (0/10)	0 (0/10)	7.00±3.25	2.93±0.98	1.83±0.72	1.23±0.56	0.40±0.21	2.12±0.76	0.93±0.25
Hyperplastic epidermis										
Early stage (n=15) negative (n=13) (86.7%)	0 (0/13)	0 (0/13)	0 (0/13)	15.56±4.25*	6.59±2.45*	2.08±0.49*	1.61±0.72*	0.52±0.18*	2.21±0.84*	0.87±0.29*
positive (n=2) (13.3%)	0 (0/2)	0 (0/2)	0 (0/2)	18.2±6.43* ***	7.72±1.48* ***	2.70±0.73* ***	1.85±0.49* ***	0.92±0.34* ***	2.95±0.72* ***	0.69±0.23* ***
Middle stage (n=15) negative (n=8) (53.3%)	0 (0/8)	0 (0/8)	0 (0/8)	17.98±5.12*	7.54±3.64*	2.36±0.45*	1.79±0.83*	0.70±0.29*	2.48±0.84*	0.84±0.19*
positive (n=7) (46.7%)	14.29** (1/7)	28.6** (2/7)	14.29** (1/7)	21.86±8.29* ***	8.98±2.98* ***	2.82±0.92* ***	2.15±0.76* ***	1.15±0.63* ***	3.09±0.72* ***	0.57±0.21* ***
Late stage (n=40) negative (n=9) (22.5%)	0 (0/9)	0 (0/9)	0 (0/9)	30.42±11.97*	9.02±4.53*	2.46±0.71*	1.89±0.51*	0.98±0.53*	2.61±0.85*	0.73±0.21*
positive (n=31) (77.5%)	19.4** (6/31)	41.9** (13/31)	38.7** (12/31)	41.29±18.4* ***	11.28±5.3* ***	3.12±0.49* ***	2.47±0.39* ***	1.56±0.74* ***	3.48±0.69* ***	0.43±0.28* ***
Total (n=70) negative (n=30) (42.9%)	0 (0/30)	0 (0/30)	0 (0/30)	20.49±8.26*	7.76±4.52*	2.39±0.71*	1.75±0.84*	0.70±0.55*	2.43±0.59*	0.81±0.37*
positive (n=40) (57.1%)	17.5 (7/40)	37.5 (15/40)	32.5 (13/40)	27.56±13.9* ***	9.32±3.56* ***	2.88±0.94* ***	2.22±0.63* ***	1.22±0.93* ***	3.16±0.73* ***	0.56±0.34* ***
Papillomatous lesions										
all-positive (n=30)	46.67** (14/30)	60** (18/30)	63.33** (19/30)	46.33±13.94*	13.58±3.70*	3.13±0.75*	3.56±0.59*	1.82±0.86*	3.64±1.56*	0.37±0.95*
Invasive carcinoma										
all-positive (n=20)	65** (13/20)	80** (16/20)	85** (17/20)	72.33±5.94*	16.60±3.25*	4.12±0.86*	6.21±1.60*	3.32±0.96*	4.59±1.04*	0.14±0.66*
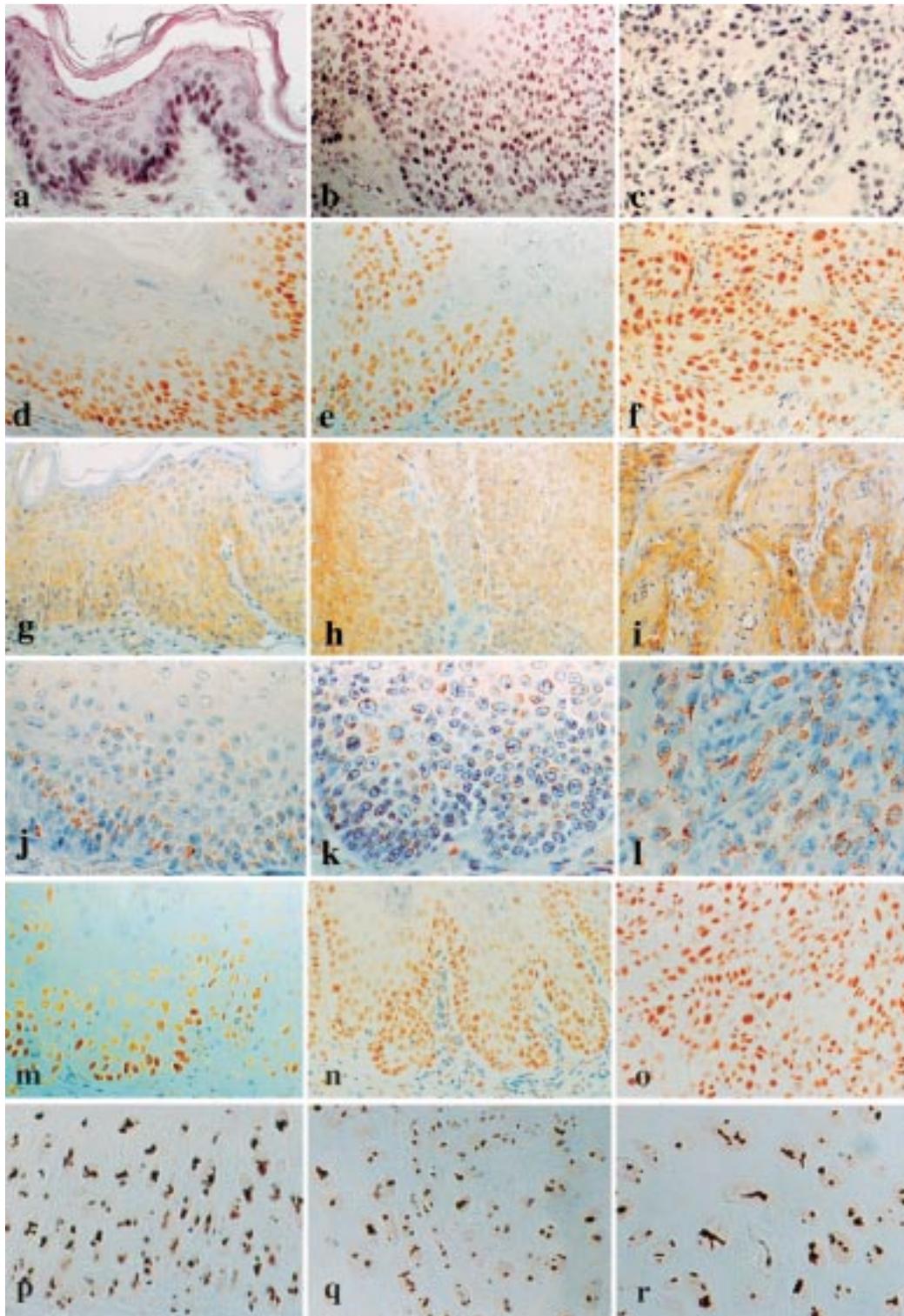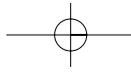
Fig. 1 - Immunohistochemical staining and AgNORs staining. Hyperplastic epidermis (a-p), papillomatous lesion (b-q), and invasive carcinoma (c-r). DNA-instability test (a, b, c) (x100), p53 (d, e, f) (x100), VEGF (g, h, i) (x100), DFF45 (j, k, l) (x100), PCNA (m, n, o) (x100), AgNORs (p, q, r) (x200).
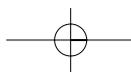
cided with that of DNA-instability test-positive cells. The percentages of DFF45-positive cells among DNA-instability test-positive foci in early, middle and late-stage hyperplastic epidermis were 0 (0/2), 14.3 (1/7), and 38.7 (12/31)%, respectively, and DFF45-positive cells were distributed in 3-4 layers of the basal side (Fig. 1j). In papillomatous lesions and invasive carcinoma lesions, the percentages of DFF45-positive lesions were 63.3 (19/30) and 85 (17/20)%, respectively, and the distribution of the DFF45-positive cells showed diffuse spread throughout the entire lesion (Fig. 1k, l). Differences in tthe values between various tissue types were significant (Table I).

(e) PCNA immunohistochemistry

The distribution of cycling cells positively stained for PCNA in normal epidermis was well defined in the basal layer and sporadic with preserved polarity of cell proliferation and cytodifferentiation (PCNA-index, 7.0%). In hyperplastic epidermis with a negative DNA-instability test, the distribution of PCNA-positive cells was slightly wider, occupying the basal and suprabasal layers (PCNA-index, 15.6%). In hyperplastic epidermis with foci of positive DNA instability, the distribution of PCNA-positive cells coincided with that of DNA instability positive-cells indicating disturbed polarity of cytodifferentiation (Fig. 1m). The percentage of PCNA-positive cells in the DNA-instability test-positive foci in hyperplastic epidermis at the early, middle and late-stages were 18.2, 21.9 and 41.3%, respectively (Table I). The values for papillomatous lesions and invasive carcinoma lesions were 46.3 and 72.3%, respectively (Table I), and these PCNA-positive cells were spread throughout the entire lesions with loss of polarity of cytodifferentiation (Fig. 1n, o). Differences in PCNA indices between different tissue types were significant (Table I).

(f) AgNORs parameters

All AgNORs parameters were significantly higher in hyperplastic epidermis, papillomatous lesions and invasive carcinoma than in normal epidermis (Fig. 1p-r, Table I). The values of invasive carcinoma were also significantly higher than those of hyperplastic epidermis and papillomatous lesions. The values for papillomatous lesions were significantly higher than those for normal epidermis and hyperplastic epidermis at all stages showing negative DNA instability. Moreover, the values of various AgNORs parameters for the DNA-instability test-positive foci of hyperplastic epidermis were significantly higher than those for the DNA-instability test-negative foci, and the values for late-stage hyperplastic epidermis were also significantly higher than those for the early and middle-stage hyperplastic epidermis (Table I).

(g) Immunohistochemical double staining for CD 34 and PCNA

The percentages of PCNA-positive vascular endothelial cells in the neighboring foci with positive DNA instability in early, middle and late-stage hyperplastic epidermis were 7.7, 9.0 and 11.3%, respectively. Differences in these percentages between these lesions were statistically significant. Moreover, PCNA indices of vascular endothelial cells for hyperplastic epidermis at different stages at foci with a positive DNA instability were all significantly higher than foci with a negative DNA-instability test. PCNA indices of vascular endothelial cells for papillomatous lesions and invasive carcinoma were 13.6 and 16.6%, respectively, and the difference between these two values, and differences between these values and those of different stages of hyperplastic epidermis described above were significant (Fig. 2a, b, Table I).

Wounding experiments

The labeling index of stromal vascular endothelial cells in small (diameter 0.5 cm), middle (diameter 1.0 cm), and large (diameter 1.5 cm) papillomatous lesions were determined on the autoradiographs prepared by flash labeling with $_3$H-TdR, before and 24 h after cut-wounding, and the results are summarized in Fig. 3. The degree of reactive enhancement of endothelial cell proliferative activity was higher in small papillomatous lesions than in large papillomatous lesions, and weak reactivity was noted in the latter only at the top zone of capillaries. In invasive carcinoma lesions, the labeling index of vascular endothelial cells was 10%, and the value decreased markedly to 3% and 0.5% at 10 and 20 days after cut-wounding, respectively. At 10 days after wounding, many cancer cells showed coagulative necrosis, although they still remained in the perivascular areas in several layers (Fig. 2c). However, at 20 days after wounding, only a small number of cancer cells remained in the perivascular areas and were limited to one or two layers (Fig. 2d).
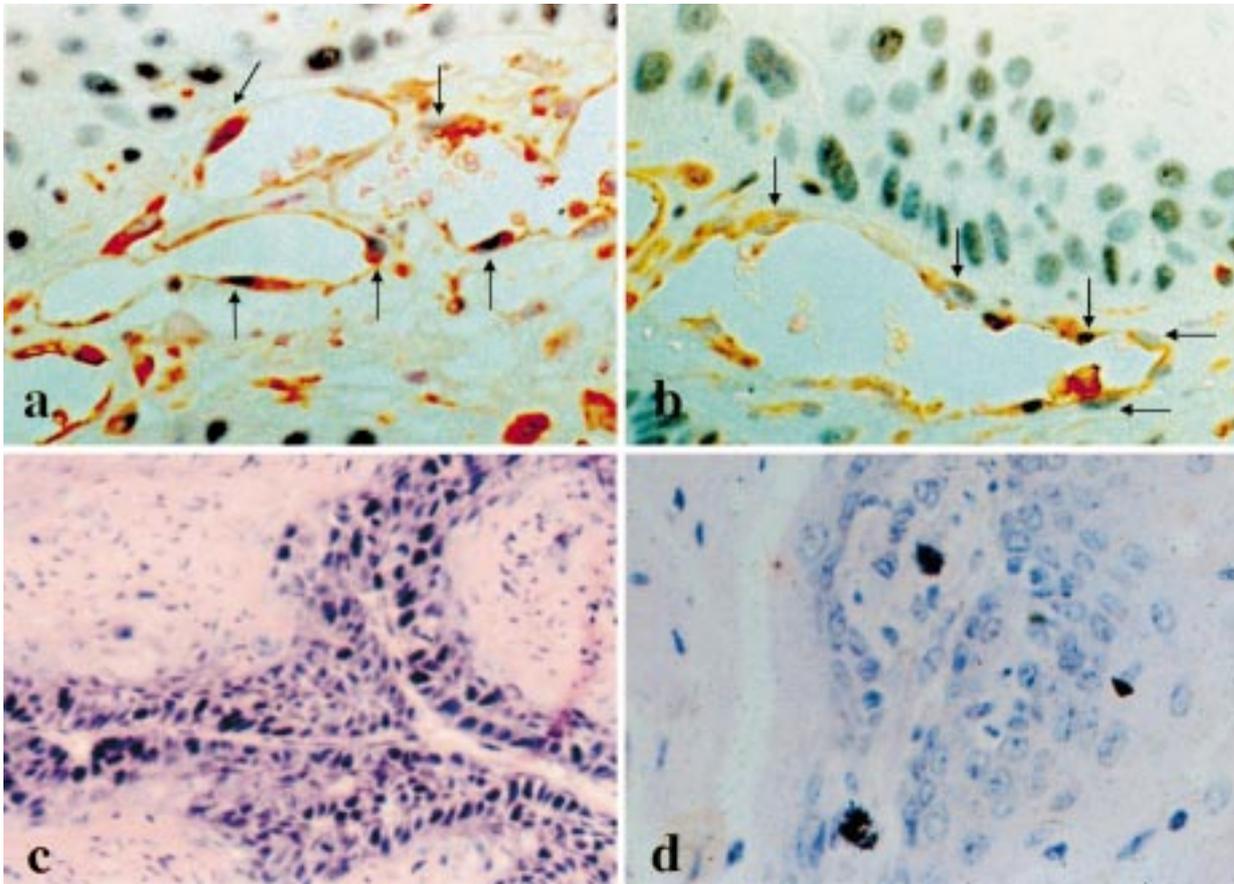
Fig. 2 - Immunohistochemical double staining for CD34 and PCNA. The arrow (→) indicates PCNA-positive endothelial cells (a, b), a; papillomatous lesion (x132), b; invasive carcinoma (x160), and autoradiographs of flash labelling with $_3$H-TdR at 10 days (c) and 20 days (d) after cut-woundings to invasive carcinomas (c: x100; d: x132).

DISCUSSION

As described in the introduction, Fukuda et al (1986, 1993) reported that the DNA-instability test is a specific marker of malignancy irrespective of epithelial or mesenchymal origin. Based on this finding, they developed the method of differential staining of malignant cells with acridine orange or anti-single-stranded DNA antiserum based on the production of denatured single-stranded DNA in cancer cells as compared to normal cells after acid hydrolysis. Based on the degree of DNA instability by acid hydrolysis, as revealed in the present study, 13.3% of early, 46.7% of middle and 77.5% of late-stage hyperplastic epidermis, and 100% of papillomatous lesions could be categorized as malignant similar to invasive carcinoma lesions (Fig. 1a-c). Moreover, the percentages of immunostained early and middle-

stage hyperplastic epidermis positive for p53, VEGF, DFF45, PCNA and AgNORs parameters were high in foci of positive DNA instability, and were markedly higher in late-stage of hyperplastic epidermis, papillomatous and invasive carcinoma lesions (Table I).

Wild-type p53 protein plays a significant role in negative regulation of cell proliferation by controlling the entry of mutant cells into the S phase and protecting cells against carcinogenesis (Finlay *et al.*, 1988; Hollstein *et al.*, 1991). The mutated p53 protein can be detected by immunohistochemical methods (Bartek *et al.*, 1990; McGregor *et al.*, 1992; Ro *et al.*, 1993; Ren *et al.*, 1996).

PCNA, an auxiliary protein for DNA polymerase delta, plays an important role in the initiation of cell proliferation (Bravo *et al.*, 1987; Wong *et al.*, 1987; Jaskulski *et al.*, 1988; Tsuji *et al.*, 1993; Yang *et al.*,
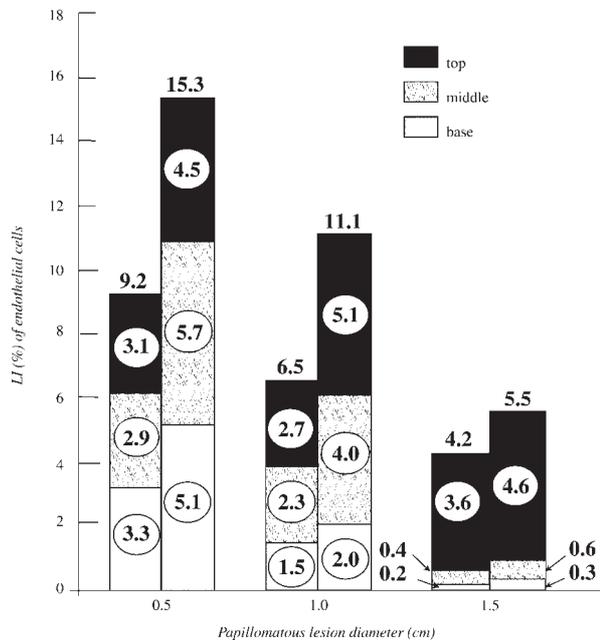
Fig. 3 - Changes of the percent labelling indexes (LI) before (left columns) and 24h after (right columns) cut-woundings given to small (diameter 0.5 cm), middle (diameter 1.0 cm), and large (diameter 1.5 cm) sized papillomatous lesions counted on the autoradiographs made by flash labelling with $_3$H-TdR.

1993). Prescreening with the monoclonal anti-PCNA antiserum used in the present study revealed that it reacts with all proliferating cells but not with mature cells and G0 cells. We defined the percentage of PCNA positive cells as the PCNA-index, and this seems to reflect the relative size of the proliferating cell fraction or growth fraction.

Nucleolar organizer regions (NORs) are chromosomal regions where genes for the major ribosomal RNA (18s, 5-8s and 28s) are located (Jordan, 1984). Several studies have shown that the size and number of AgNORs reflect the capacity of cell proliferation (Reeves *et al.*, 1984; Dervan *et al.*, 1989; Trere *et al.*, 1991; Carmen *et al.*, 1992) or the degree of malignancy of neoplasms (Arden *et al.*, 1985; Crocker. 1987; Ayres *et al.*, 1988; Muscare *et al.*, 1991; Kanitakis *et al.*, 1992). Foci of positive DNA instability also showed higher proliferative activity compared to that of normal epidermis and areas of negative DNA instability as revealed by PCNA-immunohistochemistry.

Another important finding obtained by PCNA immunohistochemistry used in the present study is the loss of polarity of PCNA-positive cells especially in the areas of positive DNA instability. In normal epidermis, the PCNA-positive cells were well localized in the basal layer, but the normal distribution was more or less disturbed already in the early or middle-stage of hyperplastic epidermis, especially in the areas of positive DNA instability, and was markedly disturbed in the late stage of hyperplastic epidermis, papillomatous lesions and invasive carcinomas. This apparently indicates the presence of disturbed cell differentiation and proliferation in these lesions, reflecting again the malignant characteristics. Although foci of positive DNA-instability test were also often positive for these biomarkers, it was almost impossible to identify them as malignant based upon simple morphological criteria, because cellular atypia in these foci was often not highly pronounced, although the nuclei and nucleoli were slightly enlarged in late-stage hyperplastic epidermis and papillomatous lesions. We propose here the concept of "procancer" (not pre-cancer) as a stage in the course of carcinogenesis and cancer progression. "Procancer" lesions represent the cancer clone at the very early stage of malignancy without distinguishable morphological atypia but with a positive DNA-instability test and other biomarkers such as p53, VEGF, DFF45, PCNA and AgNORs parameters. We also define the abnormal positivities of these biomarkers including the DNA-instability test as "functional atypia", compared with the commonly defined "morphological atypia". According to these new concepts, we interpret carcinogenesis and progression in the present experiments as follows.

"Procancer" clones with a positive DNA-instability test appeared within 2 weeks after commencing topical applications of 20-MC. The number of these cells increased continuously to the middle and late-stage hyperplastic epidermis, associated with positive staining for a variety of biomarkers. In the late-stage hyperplastic epidermis, the number of "procancer" foci increased to about 50-, while that of papillomatous lesions was less than 10 per mouse. Furthermore, all cancer lesions that could be identified by morphological atypia appeared only in the preceding papillomatous lesions, and they were not found in hyperplastic epidermis.

In the present study, all papillomatous lesions were positive for the DNA-instability test, indicating that all cells of these lesions were already cancerous. Therefore, the term "papilloma" does not seem to be appropriate and should be replaced with "papillomatous lesion".

The "procancer" clones with high DNA instability would produce multiple sub-clones by enhanced proliferative activity as revealed by

increased PCNA-positivity. Mutations of p53 gene would also enhance proliferation and reduce the likelihood of apoptotic cell death of "procancer" clones. Furthermore, paracrine secretion of VEGF by "procancer" clones in hyperplastic epidermis would induce dermal neovascularization, which in turn would enhance the growth and transformation of hyperplastic epidermis to papillomatous lesion by providing sufficient levels of oxygen and nutrients. A high percentage of DFF45-positive cells especially in the late-stage hyperplastic epidermis, papillomatous lesions and invasive carcinoma lesions could be interpreted as tissue induction. By inducing DFF45 protein, the DNase activity of DFF40 is blocked as described already in the introduction, thus allowing cancer cells to avoid the DNA-fragmentation machinery immediately before the final step of apoptosis. Taken together, these new features of "procancer" and cancer clones should favor an increase in the number of descendants to accept abundant nutrients, and have a higher chance to survive by escaping the apoptotic machinery. Consequently, multiple malignant clones with invasive capabilities are produced.

Another interesting phenomenon identified in the present study is the different responses of papillomatous lesions and invasive carcinoma lesions to cut wounding (Fig. 2c, d). The paracrine secretion of VEGF by "procancer" clones in late-stage hyperplastic epidermis was also noted in papillomatous and invasive carcinomatous lesions (Fig. 1g-i, Table I). In large papillomatous lesions, the neovascularization process and regeneration of vascular endothelial cells seem to have been exhausted, although it was still present in small papillomatous lesions. However, in invasive carcinoma lesions, cell regeneration in response to cut-wounding was absent in vascular endothelial cells, and cut-wounding induced collapse of blood vessels. The massive coagulative necrosis of cancer cells by cut-wounding could be due to the loss of functions of blood vessels.

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