研　　究

Characterization of apoptotic cells induced *in vitro* in Meckel's chondrocytes by anticancer agents

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Abstract : Cell death induced in Meckel's chondrocytes *in vitro* with the anticancer drugs, etoposide and camptothecin, was examined from the viewpoint of an apoptotic event. Cell death was induced *in vitro* in Meckel's chondrocytes that were enzymatically isolated from 16-day gestation mouse embryos. The effects were assayed histologically and immunohistochemically by using the TUNEL procedure, and light and electron microscopy. Etoposide and camptothecin-treated cultures were followed by the strong appearance of TUNEL (TdT-mediated biotinylated dUTP nick end-labeling)-positive apoptotic cells; statistical analysis showed that these agents induced apoptosis significantly 12-24hrs after treatment. At an ultrastructural level, apoptotic cell death from etoposide was followed by the formation of cell blebs, chromatin condensation and the formation of apoptotic bodies. Immunostaining for p53 revealed that this protein was absent from intact chondrocytes but was continuously accelerated in the cells treated with etoposide and camptothecin. Bcl-2 was immunolocalized in intact chondrocytes at an early stage of culture, but was not detected in anticancer-treated cells. In the present study, we confirmed that cell death in Meckel's chondrocytes induced by etoposide and camptothecin is typical apoptotic cell death, and that these agents also induce apoptosis in intact chondrocytes.

Key words : Meckel's cartilage, Chondrocyte, Apoptosis, Anticancer agent, Etoposide and camptothecin

Introduction

During developmental morphogenesis of mammals, cell death occurs normally in various cells and tissues to regulate the size and shape of organs[1]. This systematic programmed cell death is known as apoptosis[1),2)] and is regulated by many apoptosis-related genes such as *p53*, *c-myc*, *c-fos*, and *bcl-2*[3)~5)]. Apoptosis is distinguished by morphological and biochemical characteristics; apoptotic cells display cell shrinkage, plasma membrane blebs, subsequent apoptotic body formation, condensation of the nuclear chromatin, and DNA fragmentation[1) 6)~8)], as shown by

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ultrastructural appearances and the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method [9].

Since genetically determined cellular program is essential for normal development and maintenance of tissue homeostasis, apoptosis has been described as physiological cell death[10]. However, recent reports have indicated that apoptosis can also be induced by a variety of cytotoxic agents including UV-irradiation, anticancer agents, and virus infections, in contrast to just physiological apoptosis[11)~14)]. There are some reports that anticancer agents including etoposide induce apoptosis in tumor cells [13), 15), 16)], but affects in normal cells are poorly understood. Recently, we have reported apoptotic cell death in Meckel's chondrocytes under the *in vitro*[17] and *in vivo*[18] conditions, but there is no report on the apoptosis in chondrocytes using chemotherapeutic agents such as etoposide and camptothecin. Thus, we investigated the cell death-induced effects of etoposide and camptothecin in normal chondrocytes, rather than in tumor cells. Moreover, if apoptosis by anticancer agents occurs in chondrocytes as a side effect, we might have little understanding of the actual situation with the apoptosis-inductive drugs, such as etoposide and camptothecin. In addition, little is known about the mechanisms of how apoptosis is induced in Meckel's cartilage.

This study aims to clarify the mechanisms of the cell death induced in Meckel's chondrocytes by camptothecin and etoposide, and further to analyze immunohistochemically whether expressions of bcl-2 and p53 are involved in such cell death. These results are expected to contribute to an understanding of cellular events as side effects on intact chondrocytes induced by anticancer agents.

Materials and Methods

1. Chondrocyte culture

Meckel's chondrocytes were obtained as in our earlier report [19]. In brief, pregnant mice at the 16th days of gestation were killed with a lethal dose of CO_2 and embryos immediately obtained. All animal experiments were performed according to the protocol filed with Iwate Medical University. Meckel's cartilage was dissected from the mandibles and chondrocytes were isolated enzymatically. The isolated chondrocytes were inoculated at a density of 1×10^4 cells in Penicylinder-cups (0.28 cm^2 ; Top Labo-ware, Osaka, Japan) placed at the center of a 35-mm dish (FALCON; Fukushima, Japan), and cultured in an alph-modified essential medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum (ICN Biomedicals Co., Ltd, Australia), 60 μg/ml kanamycin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), 0.03 mg/ml *L*-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 3 mM β-glycerophosphate (Wako) in a humidified incubator with a 5% CO_2 atmosphere. The medium was replaced every other day.

In preliminary experiments, we tried to culture at various concentrations of etoposide and camptothecin for apoptosis induction. Since the most effective concentration was at a dose of 200 μg/ml etoposide and 50 μg/ml camptothecin, in the present study, confluent cultures, which were treated at these concentrations after

one week of culture, were used in all experiments.

2. Indirect immunoperoxidase staining

P53 and bcl-2 were used to analyze cell death induced by anticancer agents.

For immunoperoxidase stainings for p53 and bcl-2, specimens were fixed with 4% paraformaldehyde for 2 hrs at room temperature and processed as previously described [20]. In brief, after specimens were thoroughly washed with phosphate-buffered saline (PBS), they treated with 3% H_2O_2 for 10 min at room temperature to inhibit endogenous peroxidase activity and were reactivated with a STUF-blocking kit (Stuf Company Ltd., USA) for 15 min at 90℃. Cultures were incubated with antibodies raised in rabbit against p53 (Santa Cruz Biotechnology, Inc, diluted in 1:100) or bcl-2 (Santa Cruz, diluted in 1:50) for 1.5 hrs at 37℃. The cells were washed three times with PBS and exposed to a 1:500 dilution of horseradish peroxidase-conjugated (HRP-conjugated) antibodies raised in goat against rabbit IgG (Cappel; Organon Teknika Corp., Durham, NC, USA) for 1 hr at 37℃. After thorough washing with PBS, they were visualized with a Sigma Fast DAB peroxidase substrate kit (Sigma Chemical Company, St. Louis, MO, USA) for 10 min and counterstained lightly with hematoxylin. To control for antibody specificity, control sections were processed similarly but without incubation with the primary antibodies.

3. TUNEL reaction

Apoptotic cells were detected immunohistochemically by the TdT-mediated biotinylated dUTP nick end-labeling (TUNEL) method [9]. The TUNEL method was processed according to our earlier detailed report [18]. In brief, all specimens for TUNEL examination were washed with PBS and fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min, and intrinsic peroxidase activity was inhibited with 3% H_2O_2 for 10 min. After washing with PBS that contained 0.1% Tween-20 (Kanto Chemical Co., Inc., Tokyo, Japan), cultures incubated with a reaction solution containing 100 unit/ml TdT and 10 nmol/ml biotinylated 16-2'-dUTP (Boehringer-Mannheim-Yamanouchi, Osaka, Japan) in TdT buffer for 1 hr at 37℃. Specimens were incubated with peroxidase-conjugated streptavidin-biotin complex (Boehringer-Mannheim) at a dilution of 1:1000 for 20 min and developed for 10 min with DAB for visualization of peroxidase activity. Samples were stained with hematoxylin, and mounted with glycerol-PBS (9:1, v/v).

4. Trypan blue staining

This staining was used to detect whether cells treated by anticancer agents either died or survived. Cultures were stained directly with 0.5% trypan blue in PBS (pH 7.2), and nucleus-positive cells were judged as dead.

5. Toluidine blue staining

For light microscopic observations, semithin sections of 1 μm thickness obtained from epoxy-resin samples for electron microscopy were stained with 0.1% toluidine blue (pH 3.0).

6. Hoechst staining

To analyze nuclear alterations by

anticancer agents, cultures were fixed with 1% glutaraldehyde in 0.05 M cacodylate buffer (pH7.2) for 10 min at room temperature and washed thoroughly with PBS. After specimens were stained with hoechst 33258 (Molecular Probes, Eugene, Oregon, USA), they were mounted with glycerol-PBS (9 : 1, v/v) and observed by a fluorescence microscope equipped with an ultraviolet filter (Carl Zeiss Co., Ltd. Oberkochen, Germany).

7. Statistical analysis of apoptotic cells

After confluent cultures were exposed for 12 hrs at a concentration of 200μg/ml etoposide and 50μg/ml camptothecin, they were harvested at 2, 4, 6, 12, and 24 hrs, fixed with 4% paraformaldehyde for 15 min, and processed for TUNEL reaction. The number of TUNEL-positive cells was calculated by using a micrometer, as the number of apoptotic cells per 1 mm^2 from five different areas at random in a dish, and the findings are expressed as means ± SD.

8. Electron microscopy

For electron microscopy, cultures were fixed in cold 2.5% glutaraldehyde (pH 7.2) for 2 - 4 hrs and post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer. After being dehydrated through a graded ethanol series, specimens were embedded in Epon 812 according to conventional procedures. Ultrathin sections were cut with a diamond knife on a LKB-8800 ultrotome (Bromma, Sweden), and were then stained with uranyl acetate and lead citrate prior to examination under a H-7100 (Hitachi, Tokyo, Japan) electron microscope.

Results

1. Etoposide treatment

(1) Trypan blue staining

The apoptotic bodies and nuclei of the chondrocytes undergoing cell death at the top of nodules were intensively stained (Fig. 1 a). However, chondrocytes expanding from cartilage nodules did not show apoptotic nuclear features.

(2) Toluidine blue staining

Apoptotic chondrocytes after treatment with etoposide could be easily detected by toluidine blue staining (Fig.1b). Apoptotic cells were characterized by irregular shapes, condensed chromatin and apoptotic bodies, and were easily distinguished from intact cells having clear oval nuclei.

(3) Hoechst staining

When nuclear changes from cell death were analyzed by hoechst staining, nuclear debris was indicated by positively stained fluorescence materials (Fig. 1 c). Such structures were identified as typical features in apoptotic chondrocytes.

(4) TUNEL reaction

TUNEL revealed that apoptosis occurred at a higher frequency in typical mature chondrocytes than that in undifferentiated cells. TUNEL-positive cells showing apoptotic cell death appeared markedly in cartilage nodules consisting of differentiated chondrocytes (Fig. 1 d).

2. Camptothecin treatment

(1) Trypan blue staining

After reaching confluence, typical round chondrocytes undergoing cell death were

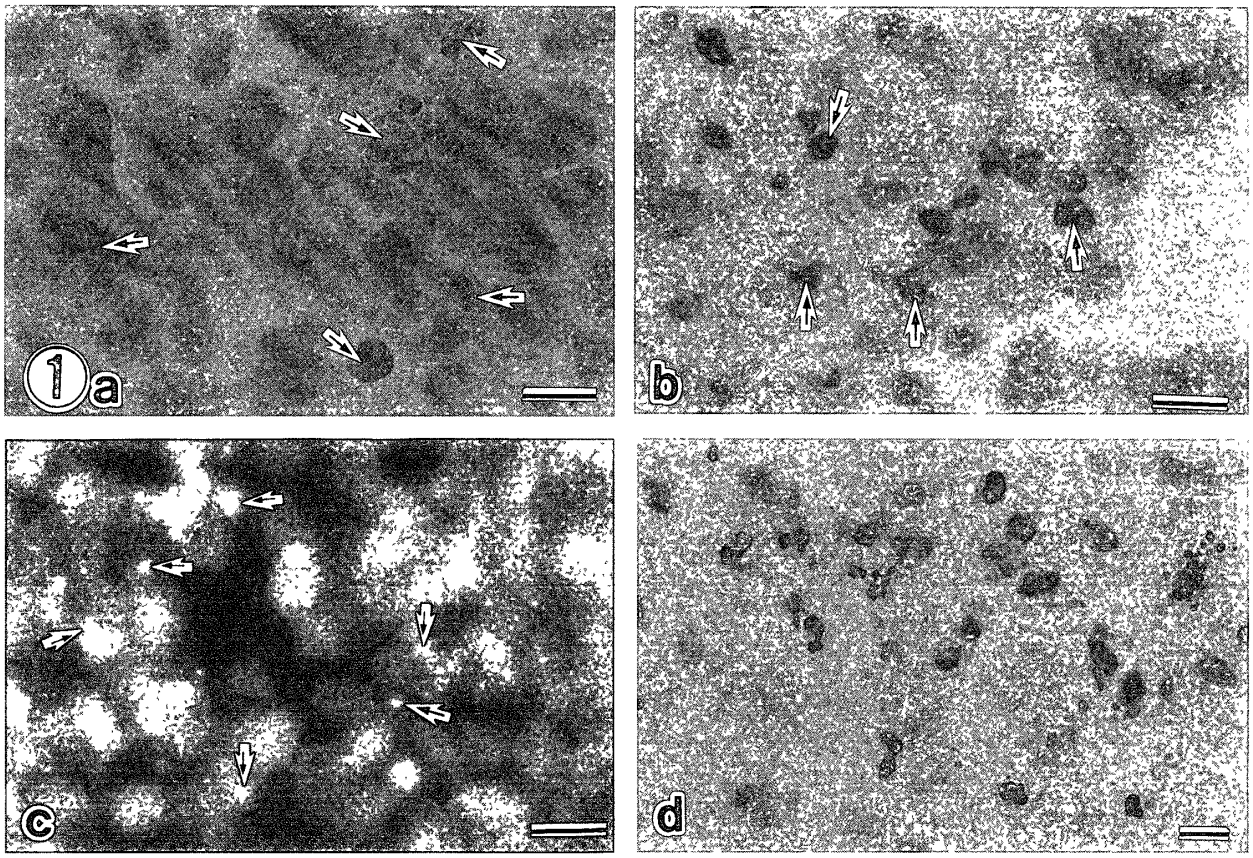

Fig 1. Chondrocytes that are shown by various histological methods after treatment for 12 hrs at a concentration of 200 μg/ml etoposide. (a) Trypan blue staining showing cell death (arrows) in the nodule-forming chondrocytes. (b) Toluidine blue staining showing nuclear alterations (arrows) by etoposide treatment. (c) Hoechst staining demonstrates the formation of nuclear fragments and apoptotic bodies (arrows) by apoptosis. (d) TUNEL reaction shows that the chondrocytes locating at the top of cartilage nodules are immunopositive predominantly. Bars = 10 μm.

well stained with trypan blue (Fig. 2 a).

(2) Toluidine blue staining

After camptothecin treatment, apoptotic chondrocytes were easily distinguished by morphological characteristics using toluidine blue staining. Apoptotic cells formed cell blebbing and contained intensively stained-apoptotic bodies (Fig. 2 b).

(3) Hoechst staining

Morphological alterations of nuclei after camptothecin exposure were evaluated by hoechst staining. Many apoptotic bodies reflecting nuclear changes were distributed in some areas of the cultures (Fig. 2 c); and apoptotic cells were easily recognized by their fragmented nuclei.

(4) TUNEL reaction

It was clearly demonstrated that chondrocytes as observed by hoechst staining and toluidine blue staining were also apoptosis-positive by TUNEL. Cells contained TUNEL-positive apoptotic bodies consisting of nuclear debris (Fig. 2 d), and were present in considerable numbers during exposure to camptothecin.

3. Statistical analyses of apoptotic cells

Frequency of the appearances of apoptotic cells after treatment by etoposide and camptothecin were analyzed statistically

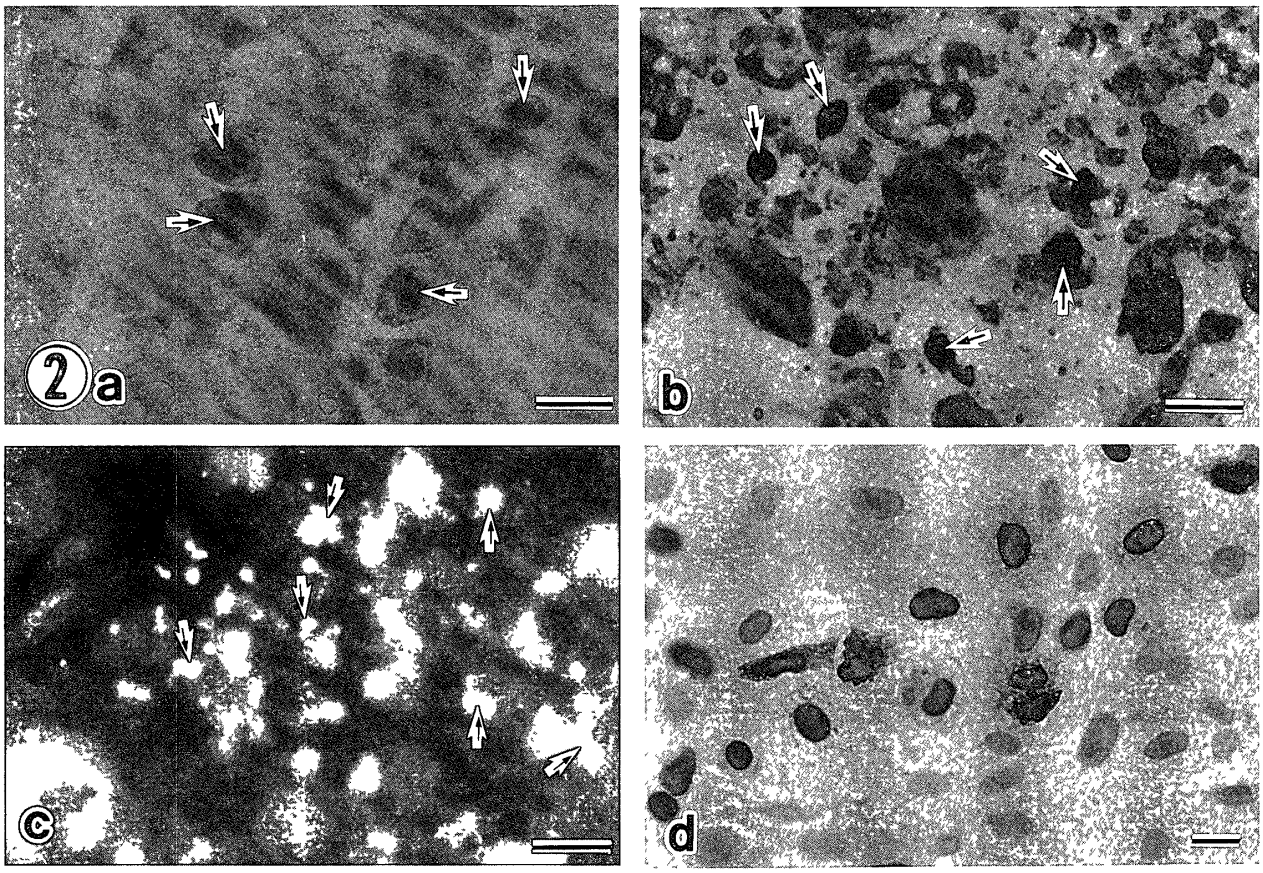

Fig 2 Apoptotic features of chondrocytes treated for 12 hrs with a concentration of 50 μg/ml camptothecin (a) Trypan blue-positive chondrocytes (arrows) showing cell degeneration are seen in the nodular structures (b) Apoptotic chondrocytes shown by staining with toluidine blue Many cells possess toluidine blue-positive condensed chromatin (arrows) (c) Hoechst staining shows nuclear changes in chondrocytes treated with camptothecin (arrows) (d) Numerous chondrocytes undergoing nuclear fragmentation are revealed as TUNEL-positive cells Bars = 10 μm

(Fig 3) After etoposide treatment, apoptotic cells showed a tendency to increase time-dependently during 2 to 6 hrs of culture, and then during 12 to 24 hrs, apoptosis was significant

Whereas apoptosis in camptothecin-treated chondrocytes occurred at a higher frequency than in etoposide treated ones during early exposure, camptothecin and etoposide induced apoptosis to similar degrees throughout 12 and 24 hrs These results indicated that the number of apoptotic cells during 2-4 hrs after etoposide and camptothecin treatment is almost consistent with that of untreated chondrocytes (approx 10-20 cells per mm^2), and further apoptotic cells significantly increase during 12 to 24 hrs

4. Electron microscopy of the etoposide-treated chondrocytes

When chondrocytes were exporsed to camptothecin and etoposide, since these agents constantly induced apoptosis, only the results obtained from etoposide-exposed cells have been described here

Morphological alterations at an early stage of apoptosis with etoposide were characterized by the formation of cytoplasmic blebbing and invaginations of nuclei Although cell organelles were well-preserved, blebs were present in almost

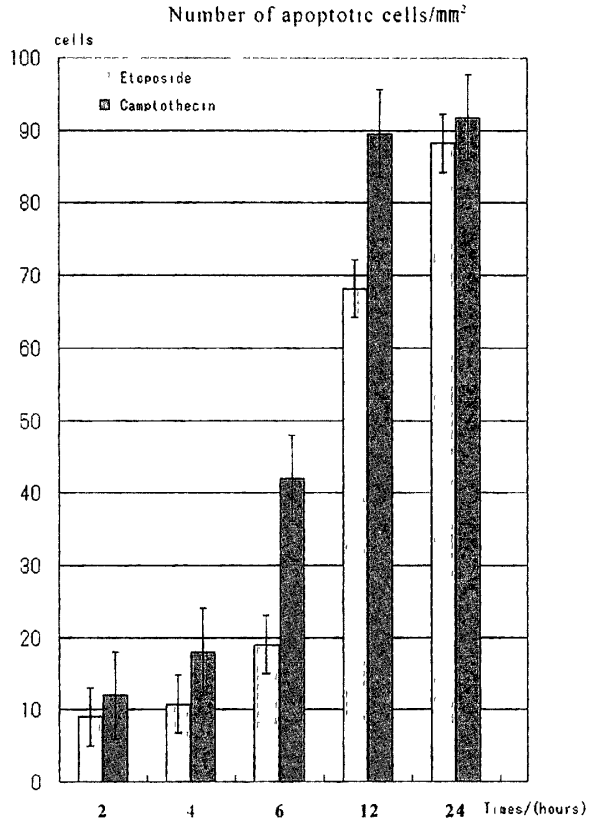

Fig 3 Statistical analyses of apoptotic cells after etoposide and camptothecin treatments The number of apoptotic cells during 2-4 hrs after etoposide and camptothecin treatment is almost consistent with that of untreated chondrocytes Both anticancer agents significantly induce apoptosis during 12-24 hrs after treatment Camptothecin appears to induce apoptosis more rapidly and to a greater degree than etoposide

cells (Fig 4 a) Next, nuclear chromatin aggregated at the peripheral zone (Fig 4 b) and nuclear changes were followed by the isolation of heterchromatin and euchromatin (Fig 4 c) The final events of nuclei were the formation of apoptotic debris consisting of isolated nuclear elements (Fig 4 d) In contrast to these dynamic nuclear modifications, organelles including rough endoplasmic reticulum and mitochondria were comparative well-preserved during the apoptotic cell processes

5. Immunostaining for bcl-2 and p53

When the intact cultures were immnunostained with the antibody against bcl-2, known as an apoptosis-suppressor protein, they revealed intensive immunoreactivity (Fig 5 a) However, immunoreactivity for bcl-2 was not detected in chondrocytes exposed to both etoposide (Fig 5 b) and camptothecin (Fig 5 c)

Expression of p53 was not detected in intact chondrocytes cultured for one week (Fig 5 d) P53 in etoposide-exposed chondrocytes was localized intranuclearly in the differentiated type of chondrocytes (Fig 5 e), while in cultures treated with camptothecin, immunoreactivity was present in chondrocytes (Fig 5 f) The immunoreactivity present showed a tendency to be less than that of etoposide-treated chondrocytes

Discussion

Recent studies have shown that apoptosis occurs in both physiological programmed cell death and in accidental cell death induced by physical and chemical agents such as virus infections, UV-irradiation, and anticancer agents[12) 14) 19)] There have been many reports on programmed cell death (apoptosis) during embryonic morphogenesis, but little is known about the apoptosis in chondrocytes *in vitro*, in particular, in Meckel's chondrocytes treated by anticancer agents Since cell death induced by etoposide and camptothecin in the present study was typical apoptotic cell death, it was shown that these agents also induce apoptosis in intact chondrocytes

Previously, we examined by TUNEL and

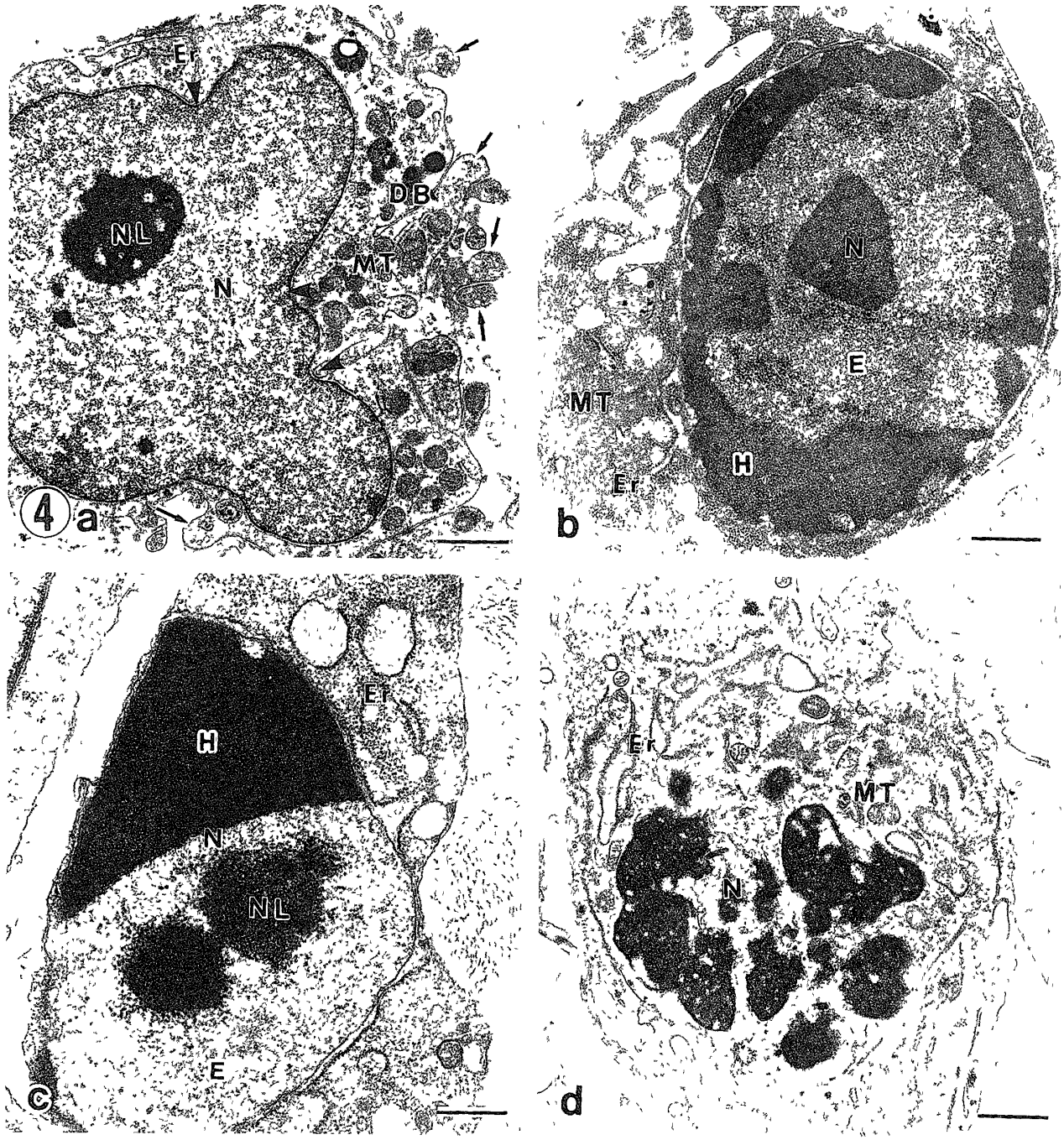

Fig 4 Electron micrographs of chondrocytes in apoptotic events (a) Initiation of apoptosis is characterized by cell buddings (arrows) of cell membranes and the invaginations of nuclear membrane (arrowheads) MT = mitochondria, Er = endoplasmic reticulum, N = nucleus, NL = nucleolus, DB = dense bodies Bar =1 5 μm (b) Cell shrinkage and clustering of nuclear chromatin occur in early apoptotic stage N = nucleus, MT = mitochondria, Er = endoplasmic reticulum, E = euchromatin, H = heterochromatin Bar =1 0 μm (c) At a subsequent stage of apoptosis, nuclear chromatin is isolated into the heterochromatin (H) and euchromatin (E) Er = endoplasmic reticulum, N = nucleus, NL = nucleolus Bar =1 5 μm (d) In final stage of apoptosis, condensed chromatin prior to the formation of apoptotic bodies is aggregated in cytoplasm but cell organelles are well-preserved in the cytoplasm N = nucleus, MT = mitochondria, Er = endoplasmic reticulum Bar =1 5 μm

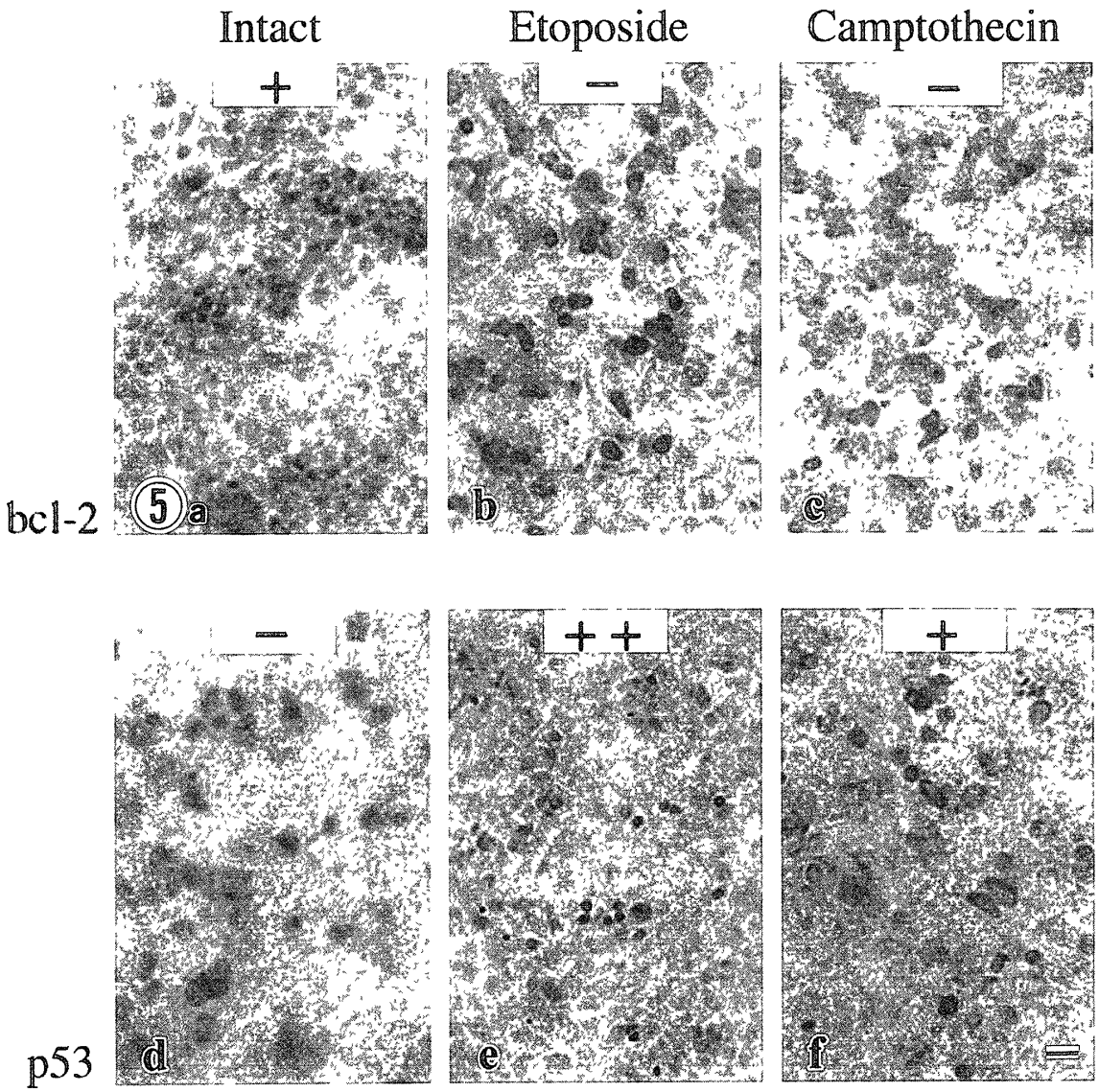

Fig 5 Immunolocalization for bcl-2 and p53 (a) Bcl-2 protein is detected in the cytoplasm of untreated chondrocytes (b) No immunoreactivity for bcl-2 is seen in etoposide-exposed chondrocytes (c) Immunoreactivity for bcl-2 is not detected in camptothecin-exposed chondrocytes (d) Immunostaining for p53 is absent in normal chondrocytes (e) P53 immunostaining showing intensive intranuclear reaction in etoposide-treated chondrocytes (f) Camptothecin-treated chondrocytes show immunopositive-reactivity for p53 Bar in f = 10 μm (all photos have the same scale)

electron microscopy whether *in vivo* Meckel's cartilage disappears during apoptosis as its ultimate fate, and indicated that apoptosis in Meckel's chondrocytes occurred not only in terminal hypertrophic chondrocytes but in any stage of chondrocytes, and that this was irrelevant to its disappearance[18] Similarly, it has been reported that *in vitro* Meckel's chondrocytes give rise to apoptotic cell death in the nodule-forming cartilage[17] Cell death in Meckel's chondrocytes is characterized by condensed nuclear chromatin, cell shrinkage and formation of apoptotic bodies, and is consistent with apoptotic evidences in other kinds of cells[1) 6) 10)] In contrast, necrotic cell death of Meckel's chondrocytes induced by lethal temperatures (50-60℃) showed a decrease in TUNEL and BrdU-positive cells and the disruption of the membrane of cell

organelles[17] Necrosis by lethal temperatures was clearly different from the morphology of apoptosis In the present study, since similar cellular events for apoptosis as described above were confirmed, we, therefore, distinguished apoptosis from cell death showing such a morphological phenotype

In general, etoposide and camptothecin have been used as chemotherapeutic agents to induce the cell death in tumor cells It is not known whether most of the drugs used in cancer therapies induce apoptosis in tumor cells There is much evidence that the cell death induced by anticancer agents is apoptotic cell death[12) 22)~24)], and that this type of cell death is also seen in camptothecin and etoposide-exposed cells It has been shown that anticancer agents such as camptothecin and etoposide which act as topoisomerase inhibitors occur DNA fragmentation and induce apoptotic cell death in tumor cells[13] Lowe et al[16] reported that etoposide induce p 53-dependant apoptosis in mouse embryonic fibroblasts Also, it is known that cisplatin induces apoptotic cell death in human ovary tumor cells, CH 1 [12] and mouse squamous carcinoma cells[23], whereas adriamycin acts as a DNA damaging agent in mouse p388 -leukemia cells[25] These reports described cell death in tumor cells, but in the present study, since similar apoptotic cell death has also occurred in normal chondrocytes, it is clear that etoposide and camptothecin induce apoptosis not only in cancer cells but also in normal cells These evidences expect to contribute to elucidation of side effects on normal cells by anticancer agents

We further examined immunohistochemical localizations for bcl-2 and p53 after treatment by anticancer agents. Bcl- 2 is expressed in various tissues, including lymphoid, epithelial, neural, endocrine, and mesenchymal types, and produces dramatic extension of cell survival[26) 27)] In general, it is known that bcl-2 suppresses apoptosis in proliferate cells[27] In the present study, the expression of bcl- 2 showed the highest immunoreactivity at an early stage of culture in intact chondrocytes, but was lost by the exposure to anticancer agents that followed (Figs 5 a–c) Thus, etoposide-treated chondrocytes lost the inhibition effects in apoptosis, and seemed to be become immunonegative for bcl-2 Since this result was also similar to camptothecin-treated chondrocytes, it might demonstrate that these agents blocked the suppressive signaling for apoptosis from bcl-2 On the other hand, p53, known as an apoptosis-facilitating factor, is enhanced by certain anticancer drugs[28] In the present study, p53 was not detected in intact Meckel's chondrocytes, but when cultured Meckel's chondrocytes were treated by etoposide and camptothecin, it was shown by immunoperoxidase staining that p53 expression increases significantly As reported by Sachs and Lotem[28] , certain anticancer drugs induce DNA-damage in tumor cells, which enhances the expression of p53, these agents, therefore, might induce apoptosis in Meckel's chondrocytes by such a mechanism

In conclusion, it was clarified that cell death induced by treatment with etoposide and camptothecin undergoes an apoptotic processes This result suggested that these anticancer agents accelerate expression of p 53, which prevents the growth of potential tumor cells and that modifies chondrocytes into an apoptotic phenotype The present

results also indicate that these agents, which are used as cancer chemotherapeutic drugs, induce apoptosis in intact chondrocytes Thus, it is necessary to further investigate from the point of view of cellular damage induced in normal cells by various kinds of anticancer drugs

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抗癌剤によって培養メッケル軟骨に誘導される アポトーシス細胞の特徴

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抄録　抗癌剤のエトポシトとカンプトセシンによってメッケル軟骨に誘導される細胞死かアポトーシスによるものか否かを検索した。細胞死は胎生16日のマウスから分離した培養メッケル軟骨にエトポシト *(200 μg/ml)* とカンプトセシン *(50 μg/ml)* によって誘導した。これらの効果はTUNEL法と光顕および電顕に加え、免疫組織化学的に解析した。エトポシト処理した培養細胞はアポトーシスの指標となる多くのTUNEL陽性細胞を示した。微細構造的にエトポシトてのアポトーシス様の細胞死は細胞性出芽、核クロマチンの濃縮、アポトーンス小体の形成によって引き続かれた。これに対し、カンプトセンンによる細胞死はエトポシトと同様にTUNEL陽性細胞の増加を示した。これらの抗癌剤処理後のアポトーンスは時間依存的に増加し、特に、12時間後から急速に増加した。P53の免疫染色ては、この蛋白は正常な培養軟骨細胞ては陰性てあったか、エトポシトとカンプトセンン処理群ては恒常的に促進された。一方、bcl-2の反応は培養初期の正常軟骨細胞に局在したか、抗癌剤処理群ては認められなかった。本研究から、エトポシトとカンプトセシンによってメッケル軟骨に誘導された細胞死は典型的なアポトーシスによる細胞死て、これらの抗癌剤は腫瘍細胞のみならす、正常な軟骨細胞にもアポトーシスを誘導することか示唆された。

キーワード　メッケル軟骨，軟骨細胞，アポトーシス，抗癌剤，
エトポシトとカンプトセンン