epithelial cells (Fukamachi 1992), to compare the growth of the epithelial cells from neighboring organs in the same culture condition. The cell number was determined by MTT assay, and the data were analyzed using Student's *t*-test, and differences were considered significant when $P < 0.05$.

RNA extraction and reverse transcription–polymerase chain reaction

The epithelial and mesenchymal tissues of forestomach, glandular stomach, duodenum and colon were obtained from 16.5 day rat fetuses, and total RNA was extracted from the tissues by Trizol (Invitrogen, San Diego, CA, USA), according the manufacturer's instructions. Approximately 1 μg of total RNA from each tissue was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and the resulting cDNA was subjected to 30 cycles of polymerase chain reaction (PCR) using ExTaq DNA polymerase (Takara, Kyoto, Japan). The following oligonucleotide primer pairs were used for the PCR reactions (expected product size in parentheses): ET-3 (477 bp), 5′-TTCTCGGGCTCACAGTGACC-3′ (sense) and 5′-GCTGGTGGACTTTATCTGTCC-3′ (antisense); ET_A (418 bp; Shigematsu *et al.* 1998), 5′-CAGATCCACATTAAGATGGG-3′ (sense) and 5′-GGAGATCAATGACCACGTAG-3′ (antisense); and ET_B (475 bp; Wang *et al.* 1996) 5′-TTCACCTCAGCAGGATTCTG-3′ (sense) and 5′-AGGTGTGGAAAGTTAGAACG-3′ (antisense). G3PDH was used as a control to compare tissues of different sizes and to compensate for varying efficiencies of extraction and reverse transcription (RT) as described previously (Matsubara *et al.* 1998). The PCR products thus generated were separated by electrophoresis on 2.0% agarose gels in 0.5x TAE buffer.

Results

Growth of colonic epithelial cells in primary serum-free culture

When fetal rat colonic tissues were treated with collagenase, epithelial and mesenchymal components could be separated with the aid of forceps. As shown in Fig. 1A, pure epithelial tissues with few contaminating mesenchymal cells could be obtained, and were used for primary culture. It was essential to seed small tissue fragments for culture, since neither single epithelial cell nor large tissue fragments would attach to the substratum. When small epithelial fragments were seeded on collagen gels, most (> 90%) of them attached to the substratum, and proliferated rapidly in the first 4 days under optimal culture conditions (see below). These epithelial cells attached closely to each other, and exhibited typical epithelial morphology with distinct margination of cell sheets (Fig. 1C–F). The epithelial nature of the cells was confirmed by the presence of cytokeratin (data not shown). Collagen gel was necessary for the attachment and proliferation of colonic epithelial cells: without collagen gels, less than 5% of the cells attached to and proliferated on plastic substratum, whereas approximately 50% of the cells attached to and proliferated on a dried collagen substratum. This is similar to the properties of duodenal epithelial cells (Ichinose *et al.* 1997). We have previously reported that collagen gel is superior to basement membrane components including laminin and type IV collagen in inducing growth of gastrointestinal epithelial cells (Ichinose *et al.* 1997). Thus in the present experiment, collagen gel was used as a substratum. The epithelial cells ceased to proliferate on days 4–5 in culture, and some cells began to degenerate on day

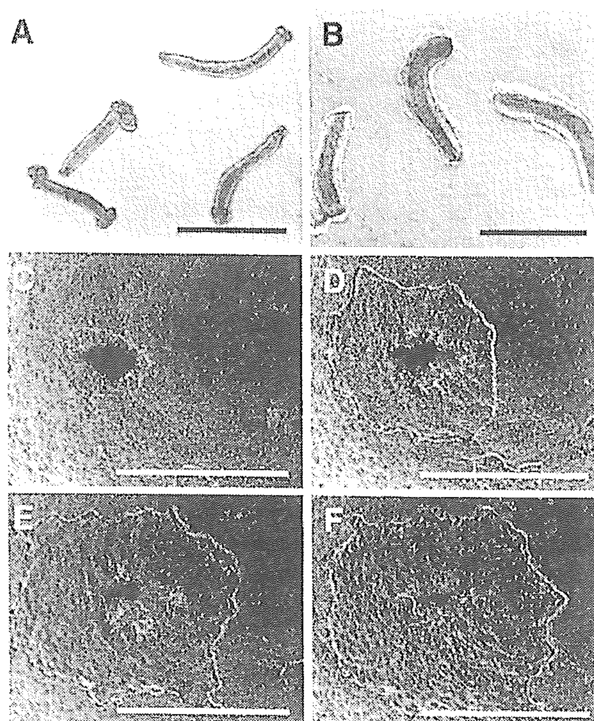

Fig. 1. Phase contrast micrographs of fetal rat colonic tissues. (A) Epithelial and (B) mesenchymal tissues just after separation by collagenase treatment. Note that no contaminating cells could be found in both tissues. (C–F) Growth of colonic epithelial cells in primary culture on days (C) 1, (D) 2, (E) 3 and (F) 4, showing sequential changes of the same tissue fragment in culture. Note that the cells grew rapidly from days 1–3, but cell growth slowed down from days 3–4. Bars, 1 mm.

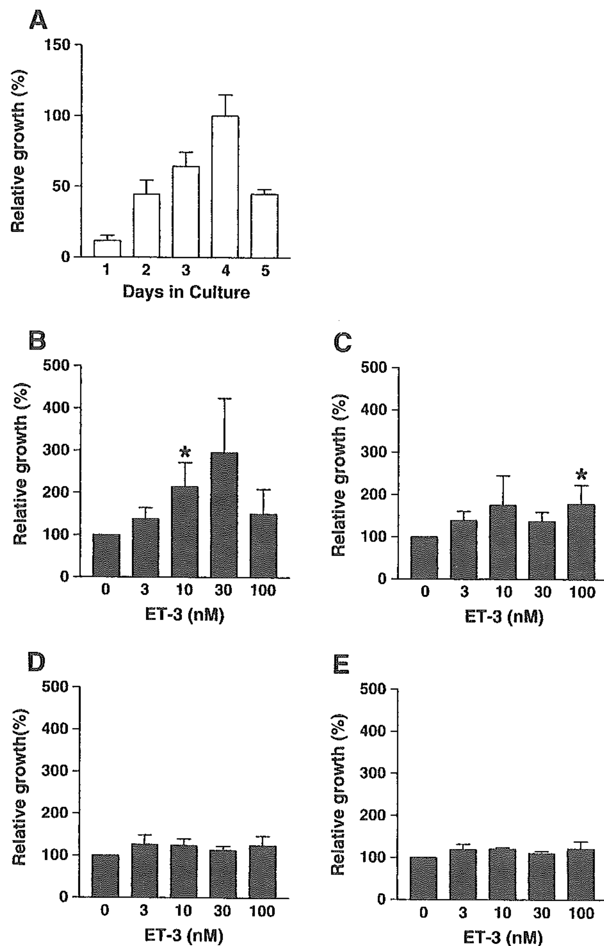

Fig. 2. (A) Growth of colonic epithelial cells in primary culture under optimal conditions. Each point represents mean ± SE of six independent experiments. (B–E) Effect of ET-3 on the growth of (B) colonic, (C) duodenal, (D) glandular stomach and (E) forestomach epithelial cells in primary culture. The cell number was determined on day 4 in culture, and data are summarized from three to six independent experiments. Asterisks indicate that values are significantly greater than controls.

5 (Fig. 2A). Attempts have been made to subculture colonic epithelial cells, but so far they have not been successful. Considering that postmitotic cells exhibit apoptosis within 4–5 days after they leave the stem cell zone *in vivo*, we speculate that it is a physiological reaction that the colonic epithelial cells ceased to proliferate on days 4–5 in culture.

The proliferation of colonic epithelial cells in culture was quantitatively measured by an MTT assay. We have previously shown that there is a good correlation between the cell number in a well and the amount of formazan produced by the cells in the range of 1×10^3 to 5×10^4 cells (Fukamachi 1992). The effect of growth factors on the proliferation of colonic epithelial cells was quantitatively examined, and optimal culture condition was determined. The cells proliferated most extensively when cultured in F-12 supplemented with EGF (10 ng/mL), insulin (30 μg/mL), cholera toxin (200 ng/mL), transferrin (10 μg/mL), hydrocortisone (2 μg/mL) and BPE (100 μg/mL; Table 1). These factors synergistically induced epithelial proliferation. This is clearly shown by Table 2, where the lack of any one of six factors severely inhibited the epithelial proliferation. Serums were not used since any lot of serum examined (a total of 10 lots, including fetal bovine serum, bovine serum and horse serum) inhibited the colonic epithelial growth (the cell number was decreased to 43 ± 18% of the controls by adding 10% serum into the complete medium).

ET-3 dose-dependently and region-specifically stimulated the growth of gastrointestinal epithelial cells

The effect of ET-3 on the growth of colonic, duodenal, glandular stomach and forestomach epithelial cells was examined in primary culture. To examine whether forestomach and glandular stomach epithelial cells respond differently to ET-3, they were cultured in the same medium optimum for glandular stomach epithelial cells. In case of duodenal and colonic epithelial cells, their response to ET-3 was compared in a medium for duodenal epithelial cells, since BPE suppressed the growth of duodenal epithelial cells (Fukamachi *et al.* 1995).

The cell number was determined on day 4 when it was greatest in the culture of colonic epithelial cells, and rapid cell growth was ceased in the cultures of duodenal (Fukamachi 1992) and glandular stomach (Fukamachi *et al.* 1994a) epithelial cells. ET-3 dose-dependently stimulated the growth of colonic and duodenal epithelial cells, and their numbers were increased about 3-fold and 1.8-fold by adding 30 nM and 100 nM ET-3, respectively (Fig. 2B,C). In contrast, ET-3 only weakly stimulated the growth of glandular stomach and forestomach epithelial cells (Fig. 2D,E). These results indicate that colonic epithelial cells are the most responsive to the growth-stimulating effect of ET-3, followed by duodenal and glandular stomach epithelial cells. We thus conclude that ET-3 may play an important role in controlling gastrointestinal epithelial growth in development, especially in the colon.

ET-3 *and its receptor were expressed by both mesenchymal and epithelial tissues in the developing gut*

Next, we examined whether *ET-3* and receptors for ET-3, were expressed in the fetal rat gastrointestinal

Table 1. Combinations and concentrations of growth factors which induce maximal growth of gastrointestinal epithelial cells in primary culture

Growth factors	Forestomach	Glandular stomach	Duodenum	Colon
Horse serum (%)	10	10	–	–
BPE (μg/mL)	100	100	–	100
EGF (ng/mL)	10	10	20	10
Cholera toxin (ng/mL)	300	300	200	200
Hydrocortisone (μg/mL)	1	3	3	2
Insulin (μg/mL)	–	3	30	30
Transferrin (μg/mL)	–	–	100	10

The best concentration of a factor was determined by changing its concentration while the concentration of other factors was kept constant. Data for forestomach, glandular stomach and duodenum are from Fukamachi *et al.* (1995).
–, No effect or inhibitory for epithelial growth. BPE, bovine pituitary extract; EGF, epidermal growth factor.

Table 2. Effect of depletion of a growth factor from the complete media on the growth of gastrointestinal epithelial cells in primary culture (cell numbers are expressed as percentage by regarding the cell number in complete media as 100%)

Growth factors	Forestomach	Glandular stomach	Duodenum	Colon
Horse serum	16 ± 10(5)[†]	57 ± 25(5)	–	–
BPE	54 ± 11(5)	48 ± 9(5)	–	59 ± 22(5)
EGF	75 ± 7(5)	79 ± 15(5)	34 ± 17(8)	72 ± 10(6)
Cholera toxin	61 ± 11(5)	56 ± 6(59)	28 ± 27(7)	55 ± 23(5)
Hydrocortisone	48 ± 10(7)	60 ± 16(5)	24 ± 19(9)	69 ± 28(8)
Insulin	–	85 ± 9(5)	59 ± 49(8)	58 ± 14(6)
Transferrin	–	–	37 ± 19(7)	59 ± 16(6)

[†]Mean ± SD (number of experiments). Data for forestomach, glandular stomach and duodenum are from Fukamachi *et al.* (1995).
–, No effect or inhibitory for epithelial growth. BPE, bovine pituitary extract; EGF, epidermal growth factor.

tract by RT–PCR. It was difficult to measure the concentration of ET-3 protein in the developing gastrointestinal tissues because the tissues were too small to obtain reproducible results, but reproducible results could be obtained at the level of mRNA. As shown in Fig. 3, *ET-3* and ET_B, a receptor for ET-3, were expressed by both mesenchymes and epithelia of gastrointestinal organs. In the colon and duodenum, their levels were greater in mesenchymes than in epithelia, while their levels were similar in glandular stomach and forestomach. In contrast, ET_A, a receptor for ET-1, was expressed exclusively by gastrointestinal mesenchymes, and only a slight expression could be found in the overlying epithelial cells. Considering that ET-3 significantly stimulated colonic epithelial growth, we suppose that ET-3 secreted from the colonic mesenchyme regulates the growth of overlying epithelial cells, by mediating epithelial–mesenchymal interaction.

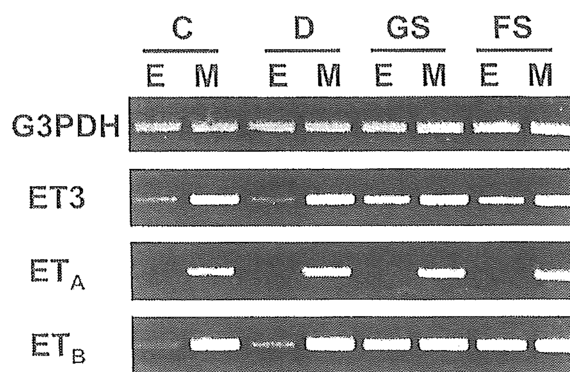

Fig. 3. Expression of *ET-3*, ET_A and ET_B genes in epithelial (E) and mesenchymal (M) tissues of colonic (C), duodenal (D), glandular stomach (GS) and forestomach (FS) tissues from 16.5-day fetal rats. Expression of mRNA for *ET-3*, ET_A, and ET_B was assessed by RT–PCR and normalized according to the expression of G3PDH.

Discussion

In the present study, we found that colonic epithelial cells from fetal rats proliferated in primary culture in a chemically defined serum-free medium in the absence of mesenchymal cells. Using this system, we quantitatively examined the effect of growth factors on the proliferation of colonic epithelial cells *in vitro*, and found that EGF, insulin, cholera toxin,

transferrin, hydrocortisone and BPE synergistically stimulated their growth while serum inhibited it. We have previously reported that EGF, insulin, cholera toxin, transferrin and hydrocortisone stimulate, and serum severely inhibits, growth of duodenal epithelial cells in primary culture (Fukamachi 1992). Thus, colonic and duodenal epithelial cells are very similar in their responsiveness to growth factors; however, there are some differences. BPE inhibits the growth of duodenal epithelial cells but stimulates that of colonic cells. Also, duodenal epithelial cells are more sensitive than colonic ones to the growth-inhibitory effects of serum. In previous studies, colonic epithelial cells were almost always grown in the presence of serum, and it has been difficult to quantify the effects of growth factors on their proliferation (Moyer & Aust 1984; Buset *et al.* 1987; Chopra *et al.* 1987; Whitehead *et al.* 1987; Kaeffer *et al.* 1997; Sugiyama *et al.* 1998). This may be due to the growth-inhibitory effect of serum. Because our culture system does not employ serum, it should be useful for future studies on the mechanisms by which colonic epithelial growth is controlled.

Using the system, we found that ET-3 significantly stimulated gastrointestinal epithelial growth. The growth-promoting activity of ET-3 has been reported on various cell types, including vascular smooth muscle cells, fibroblasts, astrocytic glial cells, osteoblastic cells, melanocytes, and mesangial cells (Battistini *et al.* 1993), but the effect of ET-3 on intestinal epithelial growth has not been examined. Using IEC-6 and IEC-18 small intestinal epithelial cell lines, Shigematsu *et al.* (1998) reported that these cells expressed *ET-1*, ET_A and ET_B, but not *ET-3*, and that exogenously added ET-1 induced a slight proliferative response in both cells. They did not examine the effect of ET-3 on the cells. Thus, our current report may be the first demonstration that ET-3 has mitogenic activity on intestinal epithelial cells.

In the present study, we found that ET-3 region-specifically stimulates the growth of gastrointestinal epithelial cells, with the greatest response from the colonic epithelial cells, followed by duodenal and glandular stomach epithelial cells. We thus speculate that there is a head-to-tail gradient in the gastrointestinal epithelial cells which controls epithelial response to ET-3, with the greatest response in the caudal region (colon) and the least response in the rostral region (glandular stomach). We have previously reported similar head-to-tail gradients which control the epithelial response to growth factors and substrata in the developing gastrointestinal epithelial cells (Fukamachi *et al.* 1995). The present result is consistent with the previous one. The nature of this gradient remains to be determined. Roberts *et al.* (1995) reported that sonic hedgehog from the endoderm plays a role in inducing a nested pattern of *Hox* gene expression in the mesoderm surrounding the hindgut, and that regionalized medoderm then instructs the endodermal tube to become the different organs of the digestive tract. Recently, canonical Wnt signaling and its inhibition have been shown to be essential for the development of gastric epithelial cells (Kim *et al.* 2005). It remains to be examined how ET-3 is involved in the specification and differentiation of gastrointestinal organs in the developing intestine.

In the present study, we found that *ET-3* and ET_B were expressed by both gastrointestinal epithelia and mesenchymes while ET_A, the receptor for ET-1, was exclusively expressed by mesenchymal tissues. That *ET-3* is expressed by intestinal mesenchymes has been repeatedly demonstrated in human and mouse fetuses (Brand *et al.* 1998; Leibl *et al.* 1999). Our finding that *ET-3* is expressed by colonic and duodenal mesenchymes is consistent with these previous studies, and together they suggest that ET-3 is an important mesenchyme-derived growth factor that controls intestinal epithelial growth in developing mammalian fetuses.

In the glandular stomach and forestomach whose epithelial growth was only scarcely stimulated by ET-3, both *ET-3* and ET_B, were expressed at a high level. Thus the level of ET_B expressed in the epithelial cells did not correlate with their responsiveness to the growth-stimulating effect of ET-3. It is possible that the growth-promoting activity of ET-3 in culture was suppressed by serum and/or BPE supplemented in the culture medium for glandular stomach and forestomach epithelial cells. It is more probable that ET-3 may play other roles such as control of epithelial differentiation in these organs. It is interesting to note that ET-1/ET_A signal transduction has recently been shown to be involved in branchial arch development (Fukuhara *et al.* 2004). It is a problem for the future how ET-3 is concerned in the differentiation and morphogenesis of the gastrointestinal organs.

It is well established that a targeted disruption of *ET-3* or ET_B results in aganglionic megacolon and pigmentary disorders in mice (Baynash *et al.* 1994; Hosoda *et al.* 1994). However, the proliferation and differentiation of colonic epithelial cells in these transgenic mice have not been fully examined mostly because they usually die several weeks after birth, before maturation. It is likely that colonic epithelial growth is altered in the transgenic mice given our finding that ET-3 affects the growth of colonic epithelial cells *in vitro*. Further studies are needed to confirm whether the colonic epithelial growth is altered in these transgenic mice in development.

We and other researchers have shown that epithelial–mesenchymal interactions play an essential role in the control of gastrointestinal epithelial growth and differentiation not only in fetal stages, but also in adults. In the present study, we present evidence that ET-3 is an important mesenchymal factor that controls colonic epithelial growth in normal development. Then ET-3 may also play a role in regulating colonic epithelial growth in various diseases including tumors. ET-1 and its receptor have been reported to play a role in colon cancer progression (Egidy *et al.* 2000). It remains to be examined whether the function of ET-3 and its receptor are altered in colon tumors.

Acknowledgement

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References

Battistini, B., Chailler, P., D'Orléans-Juste, P., Brière, N. & Sirois, P. 1993. Growth regulatory properties of endothelins. *Peptides* **14**, 385–399.

Baynash, A. G., Hosoda, K., Giaid, A. *et al.* 1994. Interaction of endothelin-3 with endothelin-B reseptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79**, 1277–1285.

Brackett, K. A. & Townsend, S. F. 1980. Organogenesis of the colon in rats. *J. Morphol.* **163**, 191–201.

Brand, M., Le Moullec, J.-M., Corvol, P. & Gasc, J.-M. 1998. Ontogeny of endothelins-1 and -3, their receptors, and endothelin converting enzyme-1 in the early human embryo. *J. Clin. Invest.* **101**, 549–559.

Buset, M., Winawer, S. & Friedman, E. 1987. Defining condition to promote the attachment of adult human colonic epithelial cells. *In Vitro Cell. Dev. Biol.* **23**, 403–412.

Chopra, D. P., Siddiqui, K. M. & Cooney, R. A. 1987. Effects of insulin, transferrin, cholera toxin, and epidermal growth factor on growth and morphology of human fetal normal colon epithelial cells. *Gastroenterology* **92**, 891–904.

Eastwood, G. L. & Trier, J. S. 1974. Epithelial cell proliferation during organogenesis of rat colon. *Anat. Rec.* **179**, 303–310.

Egidy, G., Juillerat-Jeanneret, L., Jeannin, J.-F., Korth, P., Bosman, F. T. & Pinet, F. 2000. Modulation of human colon tumor-stromal interactions by the endothelin system. *Am. J. Pathol.* **157**, 1863–1874.

Firth, J. D. & Ratcliffe, P. J. 1992. Organ distribution of the three rat endothelin messenger RNAs and the effects of ischemia on renal gene expression. *J. Clin. Invest.* **90**, 1023–1031.

Fukamachi, H. 1992. Proliferation and differentiation of fetal rat intestinal epithelial cells in primary serum-free culture. *J. Cell Sci.* **103**, 511–519.

Fukamachi, H., Ichinose, M., Ishihama, S. *et al.* 1994a. Fetal rat glandular stomach epithelial cells differentiate into surface mucous cells which express cathepsin E in the absence of mesenchymal cells in primary culture. *Differentiation* **56**, 83–89.

Fukamachi, H., Ichinose, M., Tsukada, S. *et al.* 1994b. Hepatocyte growth factor region-specifically stimulates gastro-intestinal epithelial cells in primary culture. *Biochem. Biophys. Res. Commun.* **205**, 1445–1451.

Fukamachi, H., Ichinose, M., Tsukada, S. *et al.* 1995. Growth of fetal rat gastro-intestinal epithelial cells is region-specifically controlled by growth factors and substrata in primary culture. *Develop. Growth Differ.* **37**, 11–19.

Fukamachi, H. & Takayama, S. 1980. Epithelial-mesenchymal interaction in differentiation of duodenal epithelium of fetal rats in organ culture. *Experientia* **36**, 335–336.

Fukuhara, S., Kurihara, Y., Arima, Y., Yamada, N. & Kurihara, H. 2004. Temporal requirement of signaling cascade involving endotheli-1/endothelin receptor type A in branchial arch development. *Mech. Dev.* **121**, 1223–1233.

Gabriel, S. B., Salomon, R., Pelet, A. *et al.* 2002. Segregation at three loci explains familial and population risk in Hirschsprung disease. *Nature Genet.* **31**, 89–93.

Hosoda, K., Hammer, R. E., Richardson, J. A. *et al.* 1994. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79**, 1267–1276.

Ichinose, M., Yahagi, N., Matsubara, Y. *et al.* 1997. Substratum-dependent and region-specific control of attachment and proliferation of gastrointestinal epithelial cells in primary serum-free culture. *Biochem. Biophys. Res. Commun.* **230**, 537–541.

Imagawa, W., Tomooka, Y., Yang, J., Guzman, R., Richards, J. & Nandi, S. 1984. Isolation and serum-free cultivation of mammary epithelial cells within a collagen gel matrix. In: *Cell Culture Methods for Molecular and Cell Biology*, Vol. 2 (eds D. W. Barnes, D. A. Sirbasku & G. H. Sato), pp. 127–141. Alan R Liss, New York.

Kaeffer, B., Bénard, C., Blottière, H. M. & Cherbut, C. 1997. Treatment of rat proximal and distal colonic cells with sodium orthovanadate enhances their adhesion and survival in primary culture. *Cell Biol. Int.* **21**, 303–314.

Kédinger, M., Simon-Assmann, P., Alexandre, E. & Haffen, K. 1987. Importance of a fibroblastic support for in vitro differentiation of intestinal endodermal cells and for their response to glucocorticoids. *Cell Differ.* **20**, 171–182.

Kedinger, M., Simon-Assmann, P. M., Lacroix, B., Marxer, A., Hauri, H. P. & Haffen, K. 1986. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev. Biol.* **113**, 474–483.

Kim, B.-M., Buchner, G., Miletich, I., Sharpe, P. T. & Shivdasani, R. A. 2005. The stomach mesenchymal transcription factor Barx1 specifies gastric epithelial identity through inhibition of transient Wnt signaling. *Dev. Cell* **8**, 611–622.

Leibl, M. A., Ota, T., Woodward, M. N. *et al.* 1999. Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum. *Gut* **44**, 246–252.

Levin, E. R. 1995. Endothelins. *New Engl. J. Med.* **333**, 356–363.

Matsubara, Y., Ichinose, M., Yahagi, N. *et al.* 1998. Hepatocyte growth factor activator: a possible regulator of morphogenesis during fetal development of the rat gastrointestinal tract. *Biochem. Biophys. Res. Commun.* **253**, 477–484.

Moyer, M. P. & Aust., J. B. 1984. Human colon cells: Culture and in vitro transformation. *Science* **224**, 1445–1447.

Plateroti, M., Freund, J.-N., Leberquier, C. & Kedinger, M. 1997. Mesenchyme-mediated effects of retinoic acid during rat intestinal development. *J. Cell Sci.* **110**, 1227–1238.

Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A. & Tabin, C. 1995. Sonic hedgehog is an endodermal signal inducing *Bmp-4* and *Hox* genes during induction and regionalization of the chick hindgut. *Development* **121**, 3163–3174.

Sanderson, I. R., Ezzell, R. M., Kedinger, M. *et al.* 1996. Human fetal enterocytes in vitro: modulation of the phenotype by extracellular matrix. *Proc. Natl Acad. Sci. USA* **93**, 7717–7722.

Shigematsu, T., Miura, S., Hirokawa, M. *et al.* 1998. Induction of endothelin-1 synthesis by IL-2 and its modulation of rat intestinal epithelial cell growth. *Am. J. Physiol. Gastrointest. Liver Physiol.* **275**, G556–G563.

Simo, P., Simon-Assmann, P., Arnold, C. & Kedinger, M. 1992. Mesenchyme-mediated effect of dexamethasone on laminin in cocultures of embryonic gut epithelial cells and mesenchyme-derived cells. *J. Cell Sci.* **101**, 161–171.

Sugiyama, K., Otori, K. & Esumi, H. 1998. Neoplastic transformation of rat colon epithelial cells by expression of activated human K-ras. *Jpn. J. Cancer Res.* **89**, 615–625.

Tsukada, S., Ichinose, M., Yahagi, N. *et al.* 1998. Induction of precocious pepsinogen synthesis by glucocorticoids in fetal rat gastric epithelia in organ culture: Importance of mesenchyme for epithelial differentiation. *Differentiation* **62**, 239–247.

Wang, X., Douglas, S. A., Louden, C., Vickery-Clark, L. M., Feuerstein, G. Z. & Ohlstein, E. H. 1996. Expression of endothelin-1, endothelin-3, endothelin-converting enzyme-1, and endothelin-A and endothelin-B receptor mRNA after angioplasty-induced neointimal formation in the rat. *Circ. Res.* **78**, 322–328.

Whitehead, R. H., Brown, A. & Bhathal, P. S. 1987. A method for the isolation and culture of human colonic crypts in collagen gels. *In Vitro Cell. Dev. Biol.* **23**, 436–442.

Yanagisawa, M., Kurihara, H., Kimura, S. *et al.* 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411–415.

ORIGINAL ARTICLE

Tissue damage of different submucosal injection solutions for EMR (CME)

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Background: When choosing submucosal injection solutions for EMR, tissue damage should be considered, as well as the lesion-lifting ability. The objective of the study was to find out the potential tissue damage of submucosal injection solutions.

Methods: The submucosal injection solutions examined were the following: normal saline solution (NS), 3.75% NaCl, 20% and 50% dextrose water (DW), a glycerin solution (Glyceol; 10% glycerin with 0.9% NaCl plus 5% fructose), and two hyaluronic acid (HA) solutions (0.25% 1900 kDa/NS solution and 0.125% 1900 kDa/ Glyceol solution). Furthermore, DW with different concentrations (5%, 10%, 15%, 30%, 40%) also was examined to find out the tolerable concentration without tissue damage. A total of 2 mL of each solution per stomach were injected by endoscopy into the submucosal layer at the separate sites of 4 living minipigs. Two minipigs were euthanized after 30 minutes of endoscopic observations, and the others were euthanized after additional endoscopic observations a week after injection.

Results: There was no apparent tissue damage in NS, 5% and 10% DW, Glyceol, or two solutions of HA, whereas, hypertonic solutions, except Glyceol and 10% DW, have more or less potency of tissue damage. In 3.75% NaCl and DW with concentrations of ≥20%, considerable tissue damage was observed, which might affect resected EMR specimens and ulcer healing.

Conclusions: Use of hypertonic solutions except Glyceol is not recommended with respect to tissue damage. A combination of HA and Glyceol is the most favorable submucosal injection solution, considering tissue damage and lesion-lifting ability. (Gastrointest Endosc 2005;62:933-42.)

For the purpose of preventing perforation, fluid injection into the submucosa is commonly performed during EMR to create a fluid cushion between the lesion and the muscle layer. Although the duration of lesion lifting is crucial to achieve successful results of EMR, knowledge as to tissue damage, depending on the submucosal injection solutions, should also be important. If the submucosal injection solution we choose has a high enough property of tissue damage to destroy the resected specimens, it may be difficult to make a precise histologic diagnosis of the targeted lesion. Furthermore, tissue damage of the muscle layer may result in delayed bleeding or perforation, especially in the case of the thin gut wall, such as the large and small intestines. However, there is little knowledge about the potential tissue damage of the submucosal injection solutions we widely use in clinical practice. This study compared the tissue damage of these solutions and dextrose solutions with different concentrations, by using a living minipig stomach to find out the appropriate use of submucosal injection solutions.

See CME section; p. 949.
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MATERIAL AND METHODS

Endoscopy was carried out with standard endoscopes (GIF XQ230; Olympus Optical Co, Ltd, Tokyo, Japan; and EG2931; Pentax Corp, Tokyo, Japan) in 4 minipigs (*Sus scrofa*; Miniature Swine; Chugai Research Institute for Medical Science, Inc, Nagano, Japan) that were fasted

overnight and then placed in the left lateral decubitus position after tracheal intubation and induction with general anesthesia. A disposable 23-gauge catheter injection needle (NM-200L-0423; Olympus) was used to inject 2 mL of each solution per stomach into the submucosal layer at separate sites. After injection, endoclipping was made near the injection sites to identify the locations.

All of the solutions were mixed with a minimal volume of indigo carmine dye (approximately 0.5 mL per 10 mL of solution) so that the submucosal diffusion could be visualized. The solutions examined were the following: 0.9% NaCl (normal saline solution [NS])[1]; 3.75% NaCl (hypertonic saline solution [HS])[2]; 20% dextrose water (DW)[3]; 50% DW[4]; 10% glycerin with 0.9% NaCl plus 5% fructose (Glyceol; Chugai Pharmaceutical Co, Tokyo, Japan),[5] which is widely used in Japan for the treatment of intracranial hypertension as a formulation for intravenous drip infusion[6]; two solutions of hyaluronic acid (HA) (0.25% 1900 KDa HA solution made by a 1% 1900 KDa preparation [Suvenyl; Chugai Pharmaceutical] and NS) and 0.125% 1900 KDa HA solution made with Suvenyl and Glyceol, which have the similar ability of lesion lifting[7]; and DW with five different concentrations (5%, 10%, 15%, 30%, and 40%), which also were tested to find the tolerable concentration without tissue damage because it was thought that hypertonic solutions might have the possibility of tissue damage to some extent.

The endoscope was kept in the stomach to allow observation of the injection sites for up to 30 minutes, and two minipigs were euthanized after the observations were completed to investigate the immediate tissue damage. The other two minipigs recovered from the general anesthesia and then follow-up endoscopies were carried out a day and a week after injection. When the endoscopic observations of a week after injection were completed, the remaining two minipigs were euthanized to investigate the delayed tissue damage.

The retrieved stomachs were stretched flat on a cork board with pins and then were fixed with formalin; then the stomach was cut at the separate injection sites and embedded in paraffin. Histologic sections were made from each block and were stained with H&E, and the effect of the injections on the tissue was examined microscopically.

RESULTS

Endoscopic observation

Endoscopic observations are presented in Figure 1A to H. There was little difference among the minipigs in mucosal changes caused by the same solutions. In NS, 5% and 10% DW, Glyceol, and two HA solutions, there were no apparent mucosal changes seen by endoscopy. Hypertonic solutions, except Glyceol and 10% DW, have greater or lesser degrees of tissue damage. In 15% DW, mucosal whiteness with marginal redness at the small areas of the injection sites, not all the seeping area of the solutions, was visualized within 30 minutes. A day after the injection, the mucosal redness at the injection sites was also recognized; but, a week after injection, it was difficult to point out the injection sites by endoscopy. In HS and 20% DW, the mucosal whiteness with marginal redness at all the seeping areas of the solutions was visualized within 20 minutes. A day after injection, the mucosal redness at the all seeping areas, (in the case of HS, the central erosion was visualized) also was recognized, and, a week after injection, mucosal erosion was formed. In 30%, 40%, and 50% DW, the mucosal whiteness with marginal redness at all the seeping areas of the solutions was visualized within 15 minutes. Shallow ulceration was formed a day after injection and was persistent a week after injection.

Capsule Summary

What is already known on this topic

- Previous studies of submucosal injection solutions for EMR have evaluated only the ability to create a submucosal fluid cushion and not the potential for tissue damage.
- Recommended submucosal solutions are the following: hyaluronic acid, 50% dextrose water, and Glyceol (10% glycerin with 0.9% NaCl plus 5% fructose).

What this study adds to our knowledge

- In an animal study of available submucosal injection solutions, a mixture of high-molecular weight hyaluronic acid and Glyceol provide optimal lesion-lifting ability with minimal tissue damage.

Histologic observation

There was little difference among minipigs in tissue damage caused by the same solutions (Figs. 2A to H and 3A to D). None of NS, 5% and 10% DW, Glyceol, or two HA solutions caused any apparent tissue damage as seen by histology. In 15% DW, slight degradation of epithelial glands with mild congestion of capillary blood vessels in the superficial mucosal layer was observed in the minipigs euthanized on the day of injection, whereas, no tissue damage was observed by histology in the minipigs euthanized a week after injection. In HS and 20% DW, acute mucosal erosion with degradation of epithelial glands and congestion of capillary blood vessels was observed in the minipigs euthanized on the day of injection, and these tissue damages were persistent as mucosal erosion with fibrosis of the submucosal layer a week after injection. In 30%, 40%, and 50% DW, not only mucosal damage but also muscle damage emerged on the day of injection, and ulceration extending to the submucosal layer formed a week after injection.

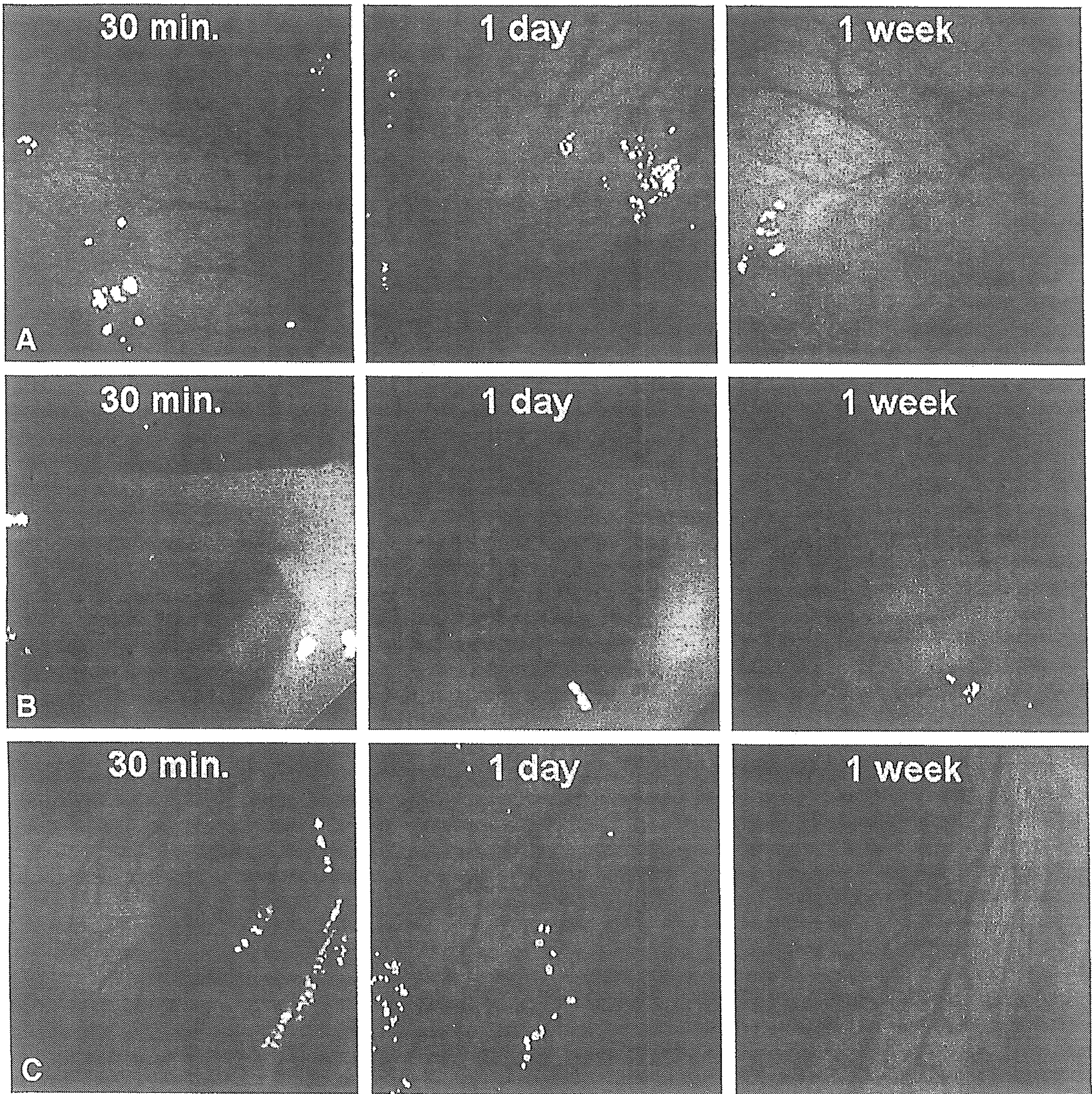

Figure 1. Endoscopic views of injection sites of various solutions. **A,** 0.9% NaCl (normal saline solution). **B,** 3.75% NaCl (hypertonic saline solution). **C,** 15% dextrose water. (*continued*)

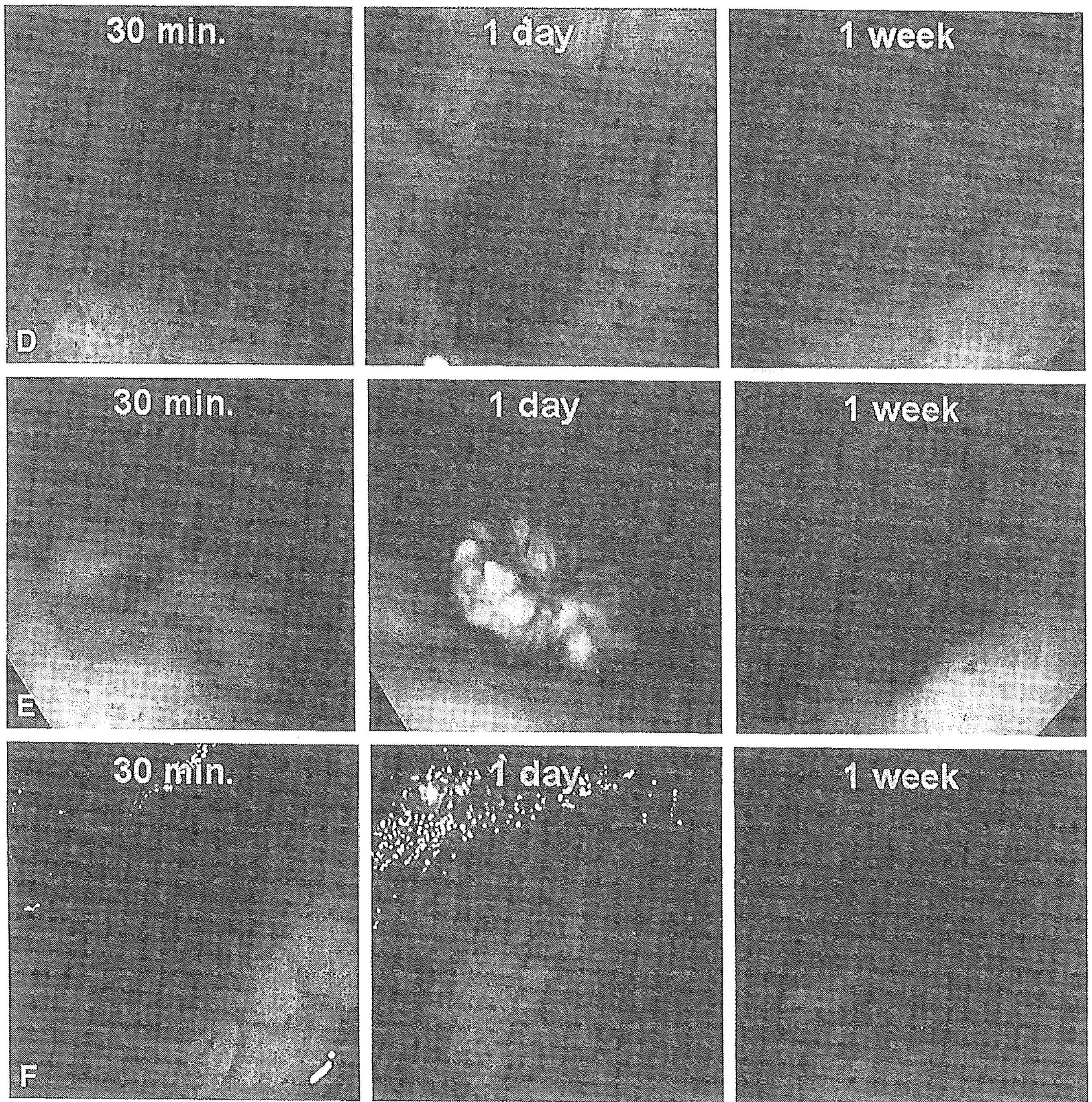

Figure 1. D, 20% dextrose water. **E,** 50% dextrose water. **F,** Glyceol (10% glycerin with 0.9% NaCl plus 5% fructose). (*continued*)

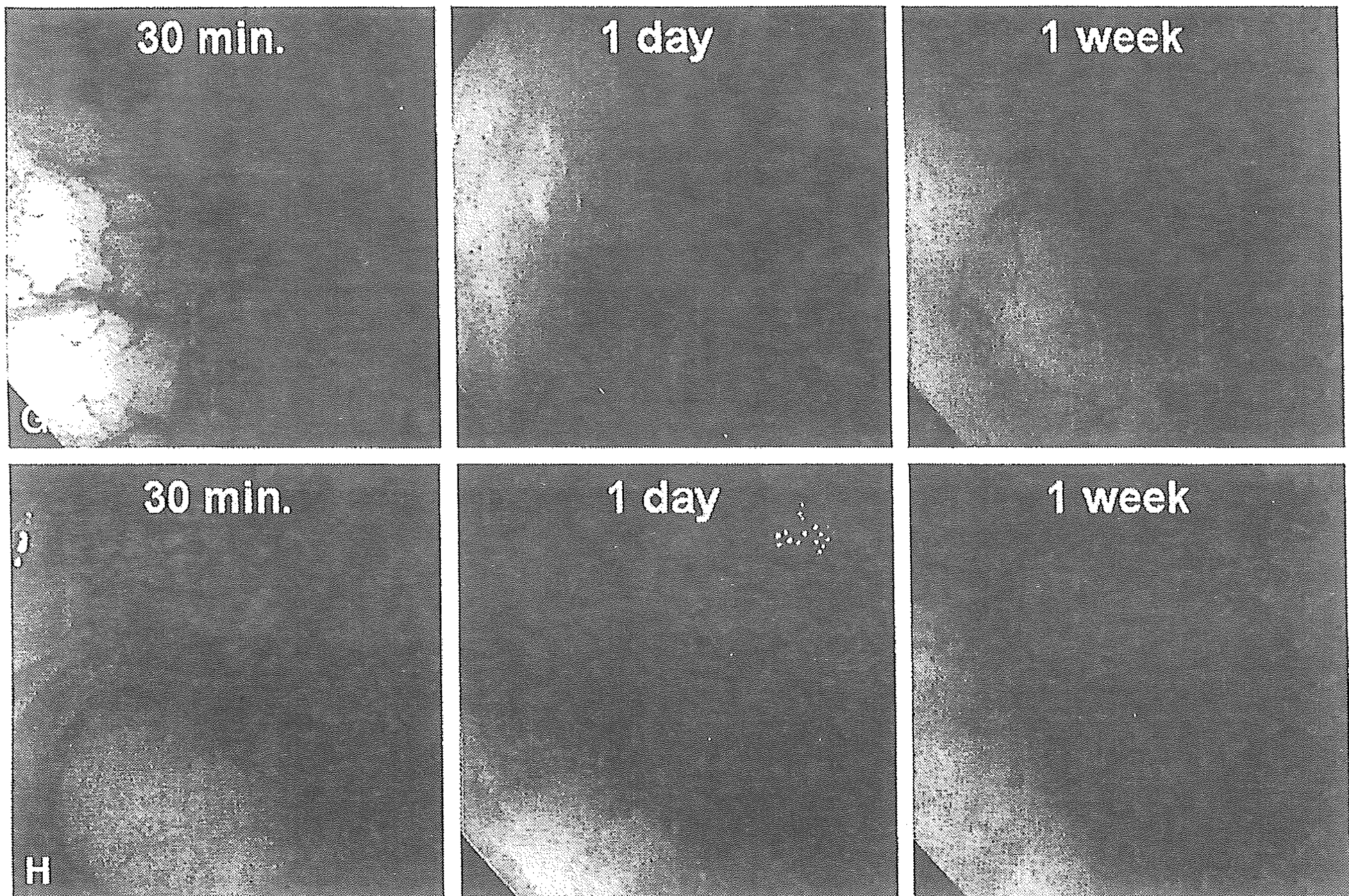

Figure 1. G, 0.25% solution of 1900 kDa hyaluronic acid with normal saline solution. **H,** 0.125% solution of 1900 kDa hyaluronic acid with Glyceol. Dextrose water with a concentration of ≥15% and hypertonic saline solution have greater or lesser degree of mucosal change. Normal saline solution, Glyceol, and two hyaluronic acid solutions have no apparent mucosal changes.

DISCUSSION

EMR has developed as a less invasive local treatment for GI tumors, which enables the preservation of the whole organ and brings about a much better quality of life for the patients with tumors.[8] In the case of the stomach, smaller intramucosal tumors without ulcer findings have been candidates for EMR until recently[9-12]; however, these days larger intramucosal tumors without ulcer findings and smaller intramucosal tumors with ulcer findings have been treated by EMR. The expansive indication of EMR has been lead by novel findings of node-negative tumors[13] and newly developed techniques in EMR,[3,14-18] called endoscopic submucosal dissection techniques. As indicated, lesions become larger or ulcerative, the frequencies of complications are increasing and relatively high complication rates have become other problems. In the use of an insulation-tipped knife for the treatment of gastric tumors, the rates of bleeding and perforation were reported as 22% and 5%, respectively.[19]

Several approaches to lessen these complications have been tried, and, among these, the most effective and simplest way to prevent complications, especially perforation, is to maintain a sufficiently thick submucosal layer for a time by endoscopic injection of fluid into the submucosa. The recent studies,[19-21] including ours, revealed that HA solutions were the best available solutions in creating the thickest fluid cushion and that hypertonic solutions tended to create higher lesion lifting than NS. These studies recommended the alternative use of hypertonic solutions in practice, because HA is much more expensive than the other solutions.

Although hypertonic fluid may be better for creating higher lesion lifting and for obtaining effective hemostasis,[22] potential tissue damage by submucosal injection may be considered, because of its high osmolality. As shown in this study, the concentration of less than 15% in DW, whose osmotic pressure is 3 times higher than the extracellular fluid, is recommended as a submucosal injection solution with respect to tolerable tissue damage, if we want to have resected specimens with good quality and avoid unexpected delayed healing of the artificial ulcers after EMR. It is speculated that Glyceol, which consists of 10% glycerin, 0.9% NaCl, and 5% fructose, has

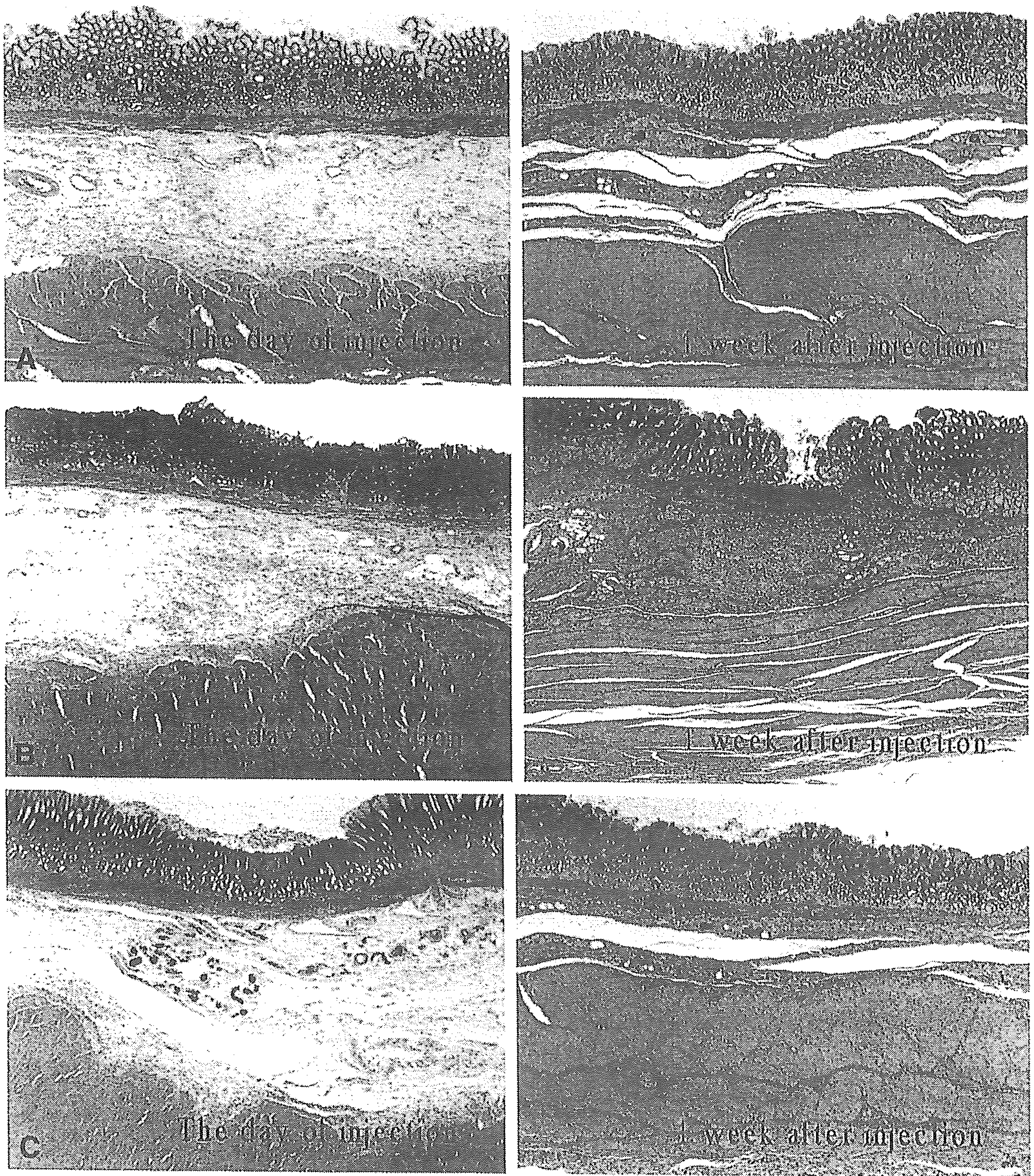

Figure 2. Histologic changes of injection sites of various solutions. **A,** 0.9% NaCl (normal saline solution) (H&E, orig. mag. ×40). **B,** 3.75% NaCl (hypertonic saline solution) (H&E, orig. mag. ×40). **C,** 15% dextrose water (H&E, orig. mag. ×40). (*continued*)

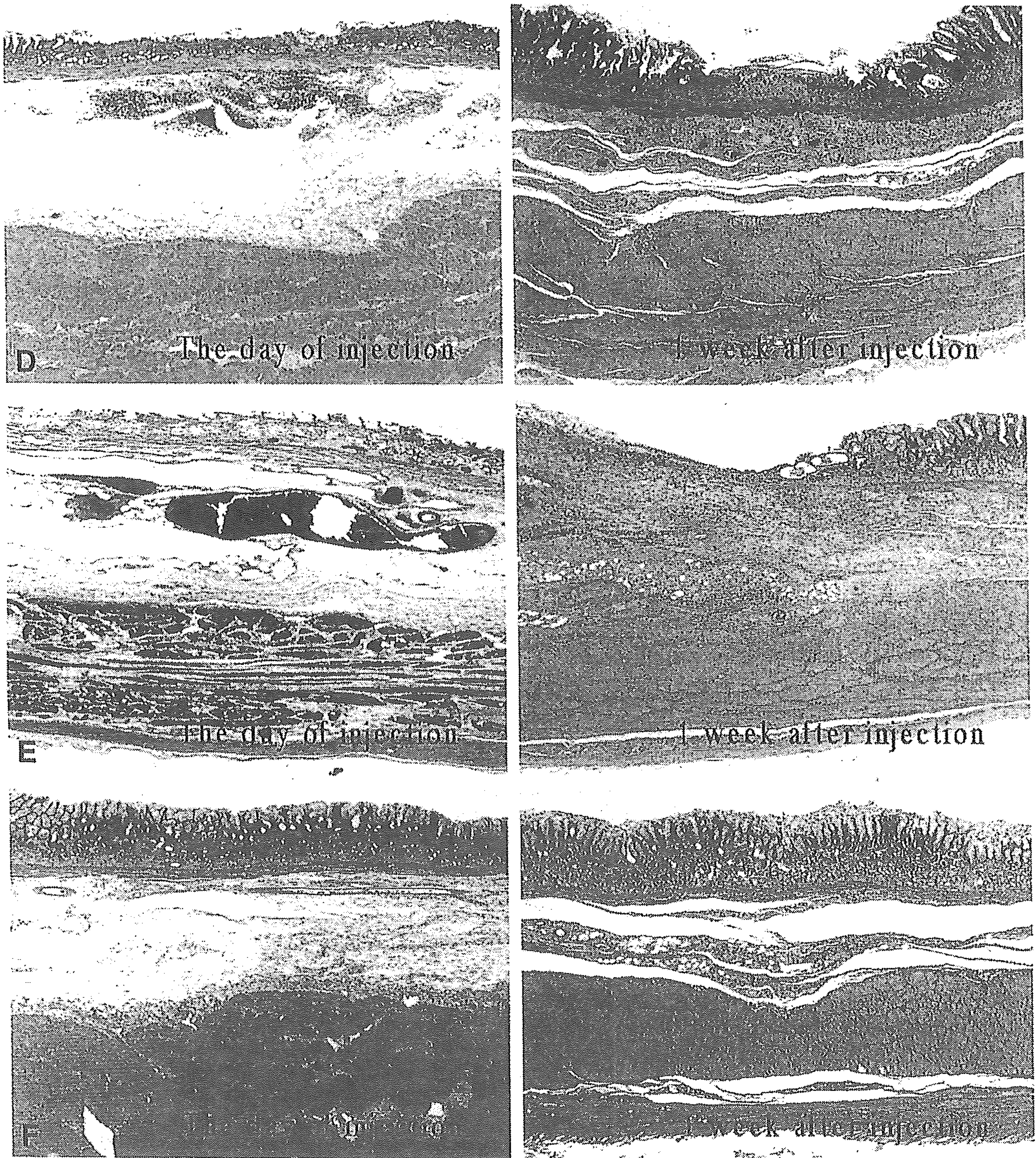

Figure 2. D, 20% dextrose water (H&E, orig. mag. ×40). **E,** 50% dextrose water (H&E, orig. mag. ×40). **F,** Glyceol (10% glycerin with 0.9% NaCl plus 5% fructose) (H&E, orig. mag. ×40). *(continued)*

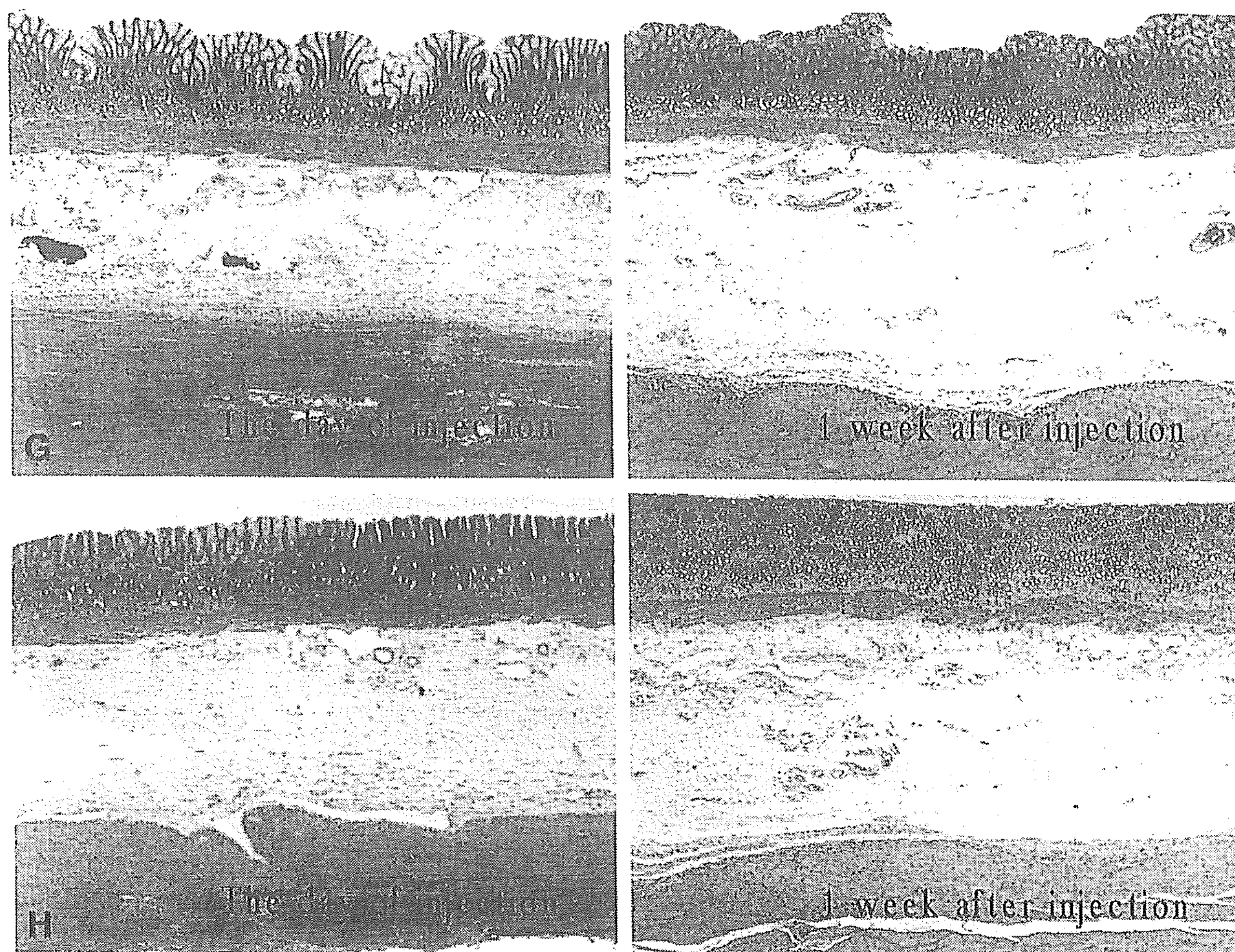

Figure 2. G, 0.25% solution of 1900 kDa hyaluronic acid with normal saline solution (H&E, orig. mag. ×40). **H,** 0.125% solution of 1900 kDa hyaluronic acid with Glyceol (H&E, orig. mag. ×40). Dextrose water with concentration of ≥15% and hypertonic saline solution have greater or lesser degree of mucosal damage, and 50% dextrose water causes tissue damage not only in the mucosal layer but also in the proper muscle layer, which is revealed by histologic assessment of the stomachs of minipigs euthanized immediately after endoscopic observations of the day of injections. Those mucosal damages do not reverse a week after injection except 15% dextrose water and mucosal erosion or ulceration is formed. Normal saline solution, Glyceol, and two hyaluronic acid solutions have no apparent mucosal changes.

no apparent tissue damage because glycerin can pass freely through the cell membrane, because the osmotic pressure difference between the inside and the outside of the cell membrane is only generated by an additional use of 5% fructose, which causes no cell destruction. On the contrary, because osmotic pressure of Glyceol is approximately 7 times higher than the extracellular fluid, Glyceol has a possibility to produce sufficient submucosal cushion.

Among available solutions, HA solutions may be the best as a submucosal injection solution with regard to tissue damage, as well as to its lesion-lifting ability. HA is a thick substance, with high viscoelasticity widely found in connective tissues. Its current approved indications in clinical practice are intra-articular injections for osteoarthritis and use in eye surgery in many countries, including Japan, Europe, and the United States. It is not antigenic or toxic to humans,[23-25] and only minor adverse effects are reported in clinical use.[26] However, the crucial disadvantage of HA solutions may be their high costs. A previous study[7] of ours revealed that a combination of high-molecular-weight HA and Glyceol is an ideal submucosal injection solution with an acceptable cost, considering the facts of increasing viscoelasticity of HA hypertonicity to create sufficient lesion-lifting effect without tissue damage.[7] If the cost of the HA solution is also unacceptable, Glyceol alone is more readily available and may be a practical alternative to HA solutions. The disadvantage of a slipping submucosal cushion by the solutions, with less lesion-lifting ability could be

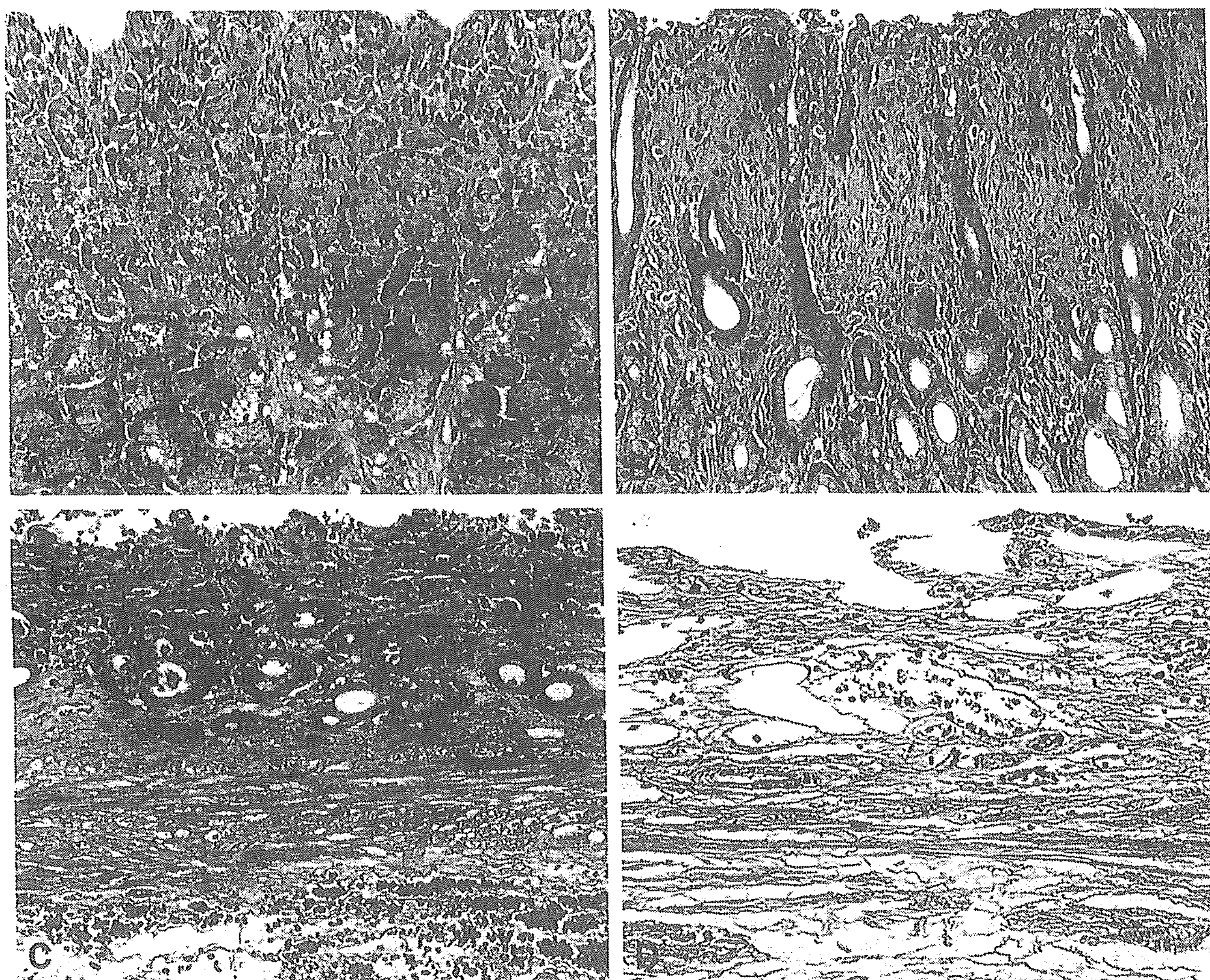

Figure 3. Histologic mucosal damages of hypertonic solutions. **A,** 3.75% NaCl (hypertonic saline solution) (H&E, orig. mag. ×200). **B,** 15% dextrose water (H&E, orig. mag. ×200). **C,** 20% dextrose water (H&E, orig. mag. ×200). **D,** 50% dextrose water (H&E, orig. mag. ×200). Partial degradation of epithelial glands and congestion of capillary blood vessels are observed in hypertonic saline solution, and 15% and 20% dextrose water. Furthermore, 20% dextrose water causes erosion of the superficial mucosal layer. In 50% dextrose water, complete degradation of epithelial cells and destruction of the mucosal structure are observed.

balanced by repeated injection of the solutions during the procedure. This also would be an alternative for the use of HA solutions or Glyceol, but there is no data available that indicates that repeated injection of a solution with less lesion-lifting ability, such as NS, achieves similar outcomes with a single injection of a solution with more lesion-lifting ability, such as HA solutions or Glyceol.

Finally, we emphasize from this study, that a submucosal injection solution should be chosen considering tissue damage as well as lesion-lifting ability to perform successful EMR with good quality. On the basis of experimental studies, including this study, a prospective clinical trial may be planned in the future, and it is necessary to find out the advantage of HA solutions or Glyceol over NS in the clinical practice.

DISCLOSURE

Koji Kashimura in a member of the Product Research Department, Kamakura Research Laboratories, Chugai Pharmaceutical Co, Ltd, Kanagawa, Japan; Toyokazu Matsuura is a member of the Chugai Research Institute for Medical Science, Inc, Nagano, Japan.

REFERENCES

1. Tada M, Shimada M, Murakami F, et al. Development of strip-off biopsy [in Japanese with English abstract]. Gastroenterol Endosc 1984;26:833-9.
2. Hirao M, Masuda K, Asanuma T, et al. Endoscopic resection of early gastric cancer and other tumors with local injection of hypertonic saline-epinephrine. Gastrointest Endosc 1988;34:264-9.

3. Yahagi N, Fujishiro M, Kakushima N, et al. Endoscopic submucosal dissection for early gastric cancer using the tip of an electrosurgical snare (thin type). Dig Endosc 2004;16:34-8.
4. Ishigooka M, Uchizawa M, Kusama K, et al. Endoscopic resection with local injection of HSE solution by direct incision of submucosa for early gastric cancer (S-ERHSE) [in Japanese with English abstract]. Endosc Dig 2002;14:1753-7.
5. Torii K, Sakai M, Kajiyama T, et al. Endoscopic aspiration mucosectomy as curative endoscopic surgery; analysis of 24 cases of early gastric cancer. Gastrointest Endosc 1995;42:475-9.
6. Tsubokawa T, Katayama Y, Ishii S. Fructose-added glycerol (Glyceol) for therapy of elevated intracranial pressure: analysis of the side effects of long-term administration in a multi-institutional trial. Neurol Res 1989;11:249-52.
7. Fujishiro M, Yahagi N, Kashimura K, et al. Different mixtures of sodium hyaluronate and their ability to create submucosal fluid cushions for endoscopic mucosal resection. Endoscopy 2004;36:584-9.
8. Roukos DH. Current advances and changes in treatment strategy may improve survival and quality of life in patients with potentially curable gastric cancer. Ann Surg Oncol 1999;6:46-56.
9. Oguro H. Progress in treatments for early gastric cancer: indication for endoscopic resection of early gastric cancer [in Japanese]. Clin Gastroenterol 1995;10:85-93.
10. Hiki N. Endoscopic mucosal resection (EMR) for early gastric cancer [in Japanese with English abstract]. Jpn J Surg 1996;97:273-8.
11. Tada M, Takekoshi T, Ashida K. Guidelines of endoscopic mucosal resection for early gastric cancer. In: Japan Gastroenterological Endoscopy Society, editors. Guidelines of gastroenterological endoscopy [in Japanese]. Tokyo: Igaku-shoin; 1999. p. 230-8.
12. The Japanese Gastric Cancer Association, editors. Guidelines for gastric cancer treatment [in Japanese]. Tokyo: Kanahara-shuppan; 2001.
13. Gotoda T, Yanagisawa A, Sasako M, et al. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. Gastric Cancer 2000;3:219-25.
14. Gotoda T, Kondo H, Ono H, et al. A new endoscopic mucosal resection procedure using an insulation-tipped electrosurgical knife for rectal flat lesions: report of two cases. Gastrointest Endosc 1999;50:560-3.
15. Yamamoto H, Sekine Y, Higashizawa T, et al. Successful en bloc resection of a large superficial gastric cancer by using sodium hyaluronate and electrocautery incision forceps. Gastrointest Endosc 2001;54:629-32.
16. Oyama T, Hotta K. Endoscopic submucosal dissection using hook knife for superficial gastric cancers [abstract]. Gastrointest Endosc 2005;61: AB234.
17. Ookuwa M, Hosokawa K, Boku N, et al. New endoscopic treatment for intramucosal tumors using an insulated-tip diathermic knife. Endoscopy 2001;33:221-6.
18. Yahagi N, Fujishiro M, Imagawa A, et al. Endoscopic submucosal dissection for the reliable en block resection of colorectal mucosal tumors. Dig Endosc 2004;16:S89-92.
19. Yamamoto H, Yube T, Isoda N, et al. A novel method of endoscopic mucosal resection using sodium hyaluronate. Gastrointest Endosc 1999;50:251-6.
20. Fujishiro M, Yahagi N, Kashimura K, et al. Comparison of various submucosal injection solutions for maintaining mucosal elevation during endoscopic mucosal resection. Endoscopy 2004;36:579-83.
21. Conio M, Rajan E, Sorbi D, et al. Comparative performance in the porcine esophagus of different solutions used for submucosal injection. Gastrointest Endosc 2002;56:513-6.
22. Hirao M, Kobayashi T, Masuda K, et al. Endoscopic local injection of hypertonic saline-epinephrine solution to arrest hemorrhage from the upper gastrointestinal tract. Gastrointest Endosc 1985; 31:313-7.
23. Nagano K, Goto S, Okabe R, et al. Acute toxicity test of sodium hyaluronate [in Japanese]. Jpn Pharmacol Ther 1984;12:5369-77.
24. Nakagawa K, Nozaki F, Sugawara H, et al. Antigenicity studies on sodium hyaluronate, part 1 [in Japanese]. Jpn Pharmacol Ther 1984;12: 141-50.
25. Nakagawa K, Nozaki F, Sugawara H, et al. Antigenicity studies on sodium hyaluronate, part 2 [in Japanese]. Jpn Pharmacol Ther 1984;12: 151-61.
26. Ueno Y, Kuramoto K, Konno N. Investigation on result of use after launch of ARTZ and ARTZ disp.: evaluation on the efficacy, safety and utility in the medication for osteoarthritis of the knee and periarthritis of the shoulder resection [in Japanese with English abstract]. Jpn Pharmacol Ther 1995;23:2151-70.

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GASTROENTEROLOGY

A combination of the *Helicobacter pylori* stool antigen test and urea breath test is useful for clinical evaluation of eradication therapy: A multicenter study

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Abstract

Background: *Helicobacter pylori* stool antigen (HpSA) test is a new tool for evaluating the *H. pylori* infection. The present study was carried out to investigate the clinical usefulness of the HpSA test in the evaluation of eradication therapy by comparing it with the ^{13}C-urea breath test (UBT).

Methods: One hundred and five patients received eradication therapy for *H. pylori*. After more than 8 weeks, the success of the therapy was evaluated by the HpSA test and the UBT. Concordant results were regarded as a final diagnosis, but when the results were discordant, histological examination was carried out.

Results: Of the 105 patients receiving eradication therapy for *H. pylori*, 25 patients were regarded as *H. pylori* positive by the UBT and and 20 patients were regarded as *H. pylori* positive by the the HpSA test. Nine patients (8.6%) showed discordant results (seven cases with UBT(+) and HpSA(–), and two with UBT(–) and HpSA(+)). Five cases out of nine were ultimately judged as having a false-positive result of the UBT, and in these cases the UBT values were relatively low (below 10 per thousand). The final diagnostic accuracies of the UBT and the HpSA test were 94.3% (88.0–97.9%; 95% CI) and 97.1% (91.9–99.4%), respectively. When we used the HpSA test in cases with weakly positive UBT values, we were able to diagnose the correct status of *H. pylori* infection after eradication in 99% of all patients (94.8–100.0%).

Conclusion: The HpSA test is a useful tool for the evaluation of eradication therapy and a combination of the HpSA test and UBT is clinically recommended.

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Key words: eradication therapy, *Helicobacter pylori*, *Helicobacter pylori* stool antigen, ^{13}C-urea breath test.

INTRODUCTION

Helicobacter pylori eradication is a popular therapy for peptic ulcer patients in Japan. Successful eradication therapy should result in the reduction of ulcer recurrence in Japanese patients.[1] Eradication therapy has come to be used for many gastric diseases including mucosa-associated lymphoid tissue (MALT) lymphoma (MALToma).[2] Although the prevalence of infection is gradually decreasing, the clinical application and

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importance of this therapy will increase in the near future.

To evaluate the final outcome of eradication therapy, many kinds of examination techniques are applicable. Although non-invasive methods are favorable for most patients, the serological test for anti-*H. pylori* antibodies is not suitable because it takes a long-time to be normalized. It is likely that the ^{13}C-urea breath test (UBT) will become the most popular and powerful method to examine the results of eradication therapy. However, the question has been raised by some researchers as to what is the most appropriate cut-off value (2.5 or 3.5 per thousand).[3] In addition, the high cost compared with the other methods is a problem.

Recently, the *H. pylori* stool antigen (HpSA) test was established and came to be applied in clinical situations. The Maastrichet Report described the equal usefulness of the UBT and the HpSA test for the judgment of not only *H. pylori* infection but also of the success of eradication therapy.[4] In the present study, we investigated the clinical usefulness of the HpSA test for the evaluation of the success of the eradication therapy by comparing it with the UBT.

METHODS

Patients

A total of 105 patients with *H. pylori* infection (55 men, mean age 56.6 years; 65 with peptic ulcers, five with gastric MALToma, and 35 with dyspepsia) were enrolled in the present study from August 2002 to December 2003, and all gave informed consent. The ethical committee of Hiroshima University approved the protocol. All patients received a gastroscopy or an X-ray examination of the upper gastrointestinal series and were confirmed to have no serious diseases present in the stomach. No patients who had received antibiotics or proton-pump inhibitors prior to entry were included in the study. *H. pylori* infection was examined by the use of a least one of three methods; the rapid urease test, ^{13}C-urea breath test or the presence of serum IgG antibodies against *H. pylori* (E-plate, Eiken, Tokyo, Japan).

An evaluation of eradication therapy was carried out at least 8 weeks after completion of therapy that involved the administration of lansoprazole (60 mg, b.i.d.), amoxicillin (1500 mg, b.i.d.) and clarithromycin (400 or 800 mg, b.i.d.).

Helicobacter pylori stool antigen and ^{13}C-urea breath test

In all patients, the *H. pylori* stool antigen test (HpSA, Meridian Diagnostics, Cincinnati, OH, USA)[5] and ^{13}C-urea breath test (UBT; Ubit IR-200, Otsuka, Tokushima, Japan)[6] were routinely carried out.

According to the manufacturer's instructions, a pieces of fecal samples were diluted in the sample solution, and stored at room temperature until analyzed. The HpSA test used polyclonal anti-*H. pylori* antibodies absorbed to microwells as a capture antibody. Sample solution was added and followed by the reaction with enzyme-conjugated polyclonal antibody. After addition of a substrate solution, the results were read by spectrophotometry at 450/630 nm.

UBT test was carried out as previously reported.[6] Fasting patients drank 100 mg of ^{13}C urea powder with 100 mL of tap water while they were in the sitting position and then immediately rinsed their mouths with water to remove the residual drug. Patients were placed in the left lateral decubitus position for 5 min and then in the sitting position for 15 min. Breath samples were collected before the drug was given, and then 20 min after the drug was given.

The cut-off values for the HpSA test and the UBT were 0.120 (OD 450/630 nm)[5] and 2.5 per thousand (delta-$^{13}CO_2$),[6] respectively. When the HpSA test value was ≥0.100 and <0.120, the HpSA test was re-examined. In 13 cases where the UBT values were <10.0, the delta-$^{13}CO_2$ was measured not only by infrared spectrometer but also by mass spectrometry analysis. If the results of the HpSA and UBT were identical, the result was regarded as the final decision. When we had discordant results, the following histological examination was carried out to make the final decision.

Histological examination

Five biopsy specimens were taken (two from the antrum, two from the corpus and one from gastric angle) and *H. pylori* colonization and grades of gastritis were evaluated by the criteria of the updated Sydney system.[7] Specimens were stained with hematoxylin and eosin (HE) and Giemsa staining. Specimens were regarded as *H. pylori* negative when no bacteria was identified and active inflammation was absent in both specimens.[8] Two experts (MI and SK) judged the histological gastritis independently without clinical information on the patients.

RESULTS

Helicobacter pylori stool antigen and ^{13}C-urea breath test

Correlation of the UBT and the HpSA test are shown as dot plots in Figure 1. Out of all 105 patients enrolled, positive results were obtained from 25 and 20 patients with the use of the UBT and the HpSA test, respectively. Seventy-eight and 18 patients were simultaneously negative and positive by the two methods, respectively. Nine patients (8.6%) showed discordant results; seven showed UBT(+) and HpSA(−) and two showed UBT(−) and HpSA(+) (Table 1). We evaluated the UBT value using two different methods (infrared spectrometry and mass spectrometry) and confirmed the very close relationship between the two results ($r = 0.995$, $P < 0.0001$; $n = 18$).

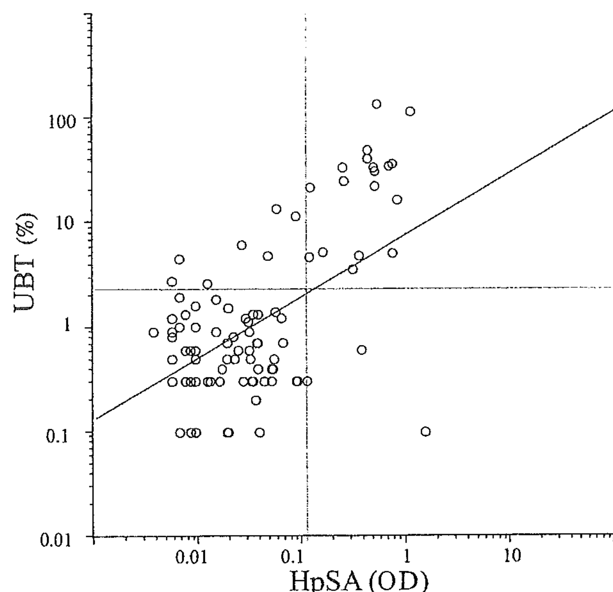

Figure 1 Correlation between the ^{13}C-urea breath test (UBT) and the *Helicobacter pylori* stool antigen (HpSA) test in patients who have undergone eradication therapy. Dot-line indicates the cut-off value in each examination. Fit line; log (UBT) = 0.586 × log (HpSA) + 0.861, $r = 0.506$, $P < 0.0001$.

Table 1 Results of ^{13}C-urea breath test and *Helicobacter pylori* stool antigen test in patients who have undergone eradication therapy

	Evaluation by HpSA		
	Negative	Positive	Total
Evaluation by UBT			
Negative	78	2	80
Positive	7	18	25
Total	85	20	105

HpSA, *Helicobacter pylori* stool antigen test; UBT, ^{13}C-urea breath test.

Histological examination in patients with discordant results

In nine cases where there were discordant results, a histological examination was carried out with gastric biopsy specimens. Results are summarized in Table 2. Histological evaluation showed results concordant with UBT test in three cases and with the HpSA test in six cases. In cases with a false positive reaction with the UBT, the delta-$^{13}CO_2$ level was relatively low (below 10 per thousand).

The diagnostic accuracy of the UBT and the HpSA test was 94.3% (88.0–97.9%; 95% CI) and 97.1% (91.9–99.4%), respectively. When we used the HpSA test in cases where there were weakly positive UBT values (≥2.5 and <10.0), we were able to diagnose the correct status of the *H. pylori* infection after eradication in 105/106 (99.1%) of all patients (94.8–100%).

Table 2 Results of ^{13}C-urea breath test, *Helicobacter pylori* stool antigen test and histology in patients with discordant results

No.	Age/sex	UBT (per thousand)	HpSA	Histology
1	74/M	neg (0.0)	pos	pos
2	52/M	neg (0.6)	pos	neg
3	72/M	pos (2.6)	neg	neg
4	71/F	pos (2.7)	neg	neg
5	38/F	pos (4.4)	neg	neg
6	84/M	pos (4.7)	neg	neg
7	66/F	pos (6.0)	neg	neg
8	43/M	pos (11.1)	neg	pos
9	44/M	pos (13.2)	neg	pos

Underlined data indicates a result that is discordant with the histological examination. HpSA, *Helicobacter pylori* stool antigen test; neg, negative; pos, positive; UBT, ^{13}C-urea breath test.

Relationship between UBT, HPSA and histological result in cases with low UBT value

Among the 25 cases with a positive result of UBT, we detected a relatively low UBT value (≥2.5 and <10.0) in 10 cases. Five cases showed simultaneously positive reactions to the HpSA test, but the remaining five were judged as *H. pylori*-negative by the HpSA test, in which histological assessment also showed the clearance of *H. pylori* and the absence of active inflammation by histological examination (case 3–7 in Table 2).

DISCUSSION

The *H. pylori* stool antigen test is a new and valuable tool for the assessment of *H. pylori* infection without an invasive procedure[9] and is reported to have higher diagnostic accuracy.[10] Because it is a simple, economical and non-invasive examination, this method has found application in patients with pediatric disease,[11–15] acute gastrointestinal bleeding[16–18] and severe statuses such as liver cirrhosis.[19] It is likely that this tool will also be invaluable for large-scale studies such as the mass survey of gastric cancer.

Recent studies have shown that the HpSA test is a useful tool, not only for the primary diagnosis of *H. pylori* infection, but also for the evaluation of eradication therapy of *H. pylori.* Odaka *et al.*[20] and Tanaka *et al.*[21] have reported the usefulness of the HpSA test in the portion of the Japanese population undergoing eradication therapy, even in the short term.[20] Vaira *et al.* also showed that the HpSA test has an identical diagnostic value compared with the UBT and emphasized the clinical usefulness of the HpSA test for the monitoring of eradication therapy.[22] Ishihara *et al.* examined the correlation between the HpSA and UBT in patients 4 weeks after their eradication therapy and they showed a close relationship between the two methods.[23]

In contrast, some reports pointed out the lesser ability of the HpSA test to evaluate eradication therapy. Perri *et al.* showed the low sensitivity of the HpSA test compared with the UBT in patients who underwent eradication.[24] To the contrary, Bilardi *et al.* reported a high incidence of false positive results in the HpSA test compared with the UBT.[25] These data are still contradictory and the reason behind these discordant results is still under discussion.

One of the major problems in this field lies in the difference in standard examinations. When we regard UBT as the standard method, false negative cases with the HpSA test will naturally increase. In some reports, histological examination has been used as the standard, and this seems appropriate. However, until now, few reports discussed the histological changes based on the international rule of the updated Sydney system. Furthermore, using only the evaluation of the presence of bacteria, we were not able to exclude the possibility of false judgment even when using complicated methods such as immunohistochemical analysis. Focus must be placed on the improvement of active gastritis in the gastric mucosa, because this is the essential goal of eradication therapy.

In the present study, we showed the clinical usefulness of the HpSA test in the evaluation of eradication therapy in Japanese patients. The HpSA test showed a relatively higher prevalence of correct diagnoses compared with UBT. We detected discordant results of the UBT and the HpSA test in 8.6% of all cases (9/105), which is identical to the previous report.[24] However, we found more than half of the cases with discordant results were a result of the false positive result in the UBT, not the false negative in the HpSA test as previously reported. To improve the accuracy of the final decision on *H. pylori* infection following eradication therapy, we followed up for a sufficient period (more than 8 weeks) after the eradication. Previous reports had shown that more than 4 or 6 months are needed after eradication to exclude the false results. In addition, we examined the histological assessment with the use of the updated Sydney system.

False-positive results of the UBT test have already been reported in other studies. In the Japanese case, Kato *et al.* reported the justification of a new cut-off value (3.5 per thousand) for the UBT.[3] In the present study, when we set the cut-off for the UBT at 3.5 per thousand, the incidence of false positives decreased (from five to three cases) without an increase in false negatives (data not shown). However, we could not exclude the possibility of false positive cases completely. In practical use, a combination of the UBT and the HpSA test must be beneficial. When there is a low-grade positive value of the UBT (less than 10 per thousand), additional examination using the HpSA test is recommended. Although the gold standard test for *H. pylori* infection is still difficult to decide, at least in studies the authors of the present study have carried out, almost all patients received an accurate diagnosis that ultimately resulted in the improvement of their active gastritis. The UBT value of 10.0, which we described as the upper limit of low-grade positive, is not based on enough evidence, and further larger-scaled examination is needed to determine the proper cut-off value.

Another problem might be a result of the difference in the methods, such as the ELISA system used in the HpSA test and the cut-off value in these examinations. Recently, HpSA ELISA using monoclonal antibodies has been established, and reported similar results to that of the polyclonal antibody which was used in the present study.[26] The difference in the status of infection between nations might also be a problem. Particularly in Japanese populations, atrophic change is very common and the amount of *H. pylori* tends to be less than in Western populations.[6]

Taken together, HpSA is a very useful and non-invasive diagnostic tool for the evaluation of eradication therapy of *H. pylori* in Japanese patients. A combination of the use of the HpSA test and the UBT is very practical in the clinical evaluation of eradication therapy of *H. pylori*. Because simultaneous examination of UBT and HpSA test contains some practical and economic issues at present, this should be discussed further.

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REFERENCES

1 Asaka M, Kato M, Sugiyama T *et al.* Follow-up survey of a large-scale multicenter, double-blind study of triple therapy with lansoprazole, amoxicillin, and clarithromycin for eradication of *Helicobacter pylori* in Japanese peptic ulcer patients. *J. Gastroenterol.* 2003; **38**: 339–47.

2 Asaka M, Satoh K, Sugano K *et al.* Guidelines in the management of *Helicobacter pylori* infection in Japan. *Helicobacter* 2001; **6**: 177–86.

3 Kato C, Sugiyama T, Sato K *et al.* Appropriate cut-off value of ^{13}C-urea breath test after eradication of *Helicobacter pylori* infection in Japan. *J. Gastroenterol. Hepatol.* 2003; **18**: 1379–83.

4 Malfertheiner P, Megraud F, O'Morain C *et al.* European *Helicobacter Pylori* Study Group (EHPSG). Current concepts in the management of *Helicobacter pylori* infection – the Maastricht 2-2000 Consensus Report. *Aliment. Pharmacol. Ther.* 2002; **16**: 167–80.

5 Matsuda M, Noda Y, Takemori Y. Utility and limitations of a method for detecting *Helicobacter pylori*-specific antigens in the stool. *J. Gastroenterol.* 2003; **38**: 222–8.

6 Chen X, Haruma K, Kamada T *et al.* Factors that affect results of the ^{13}C urea breath test in Japanese patients. *Helicobacter* 2000; 5: 98–103.

7 Dixon MF, Genta RM, Yardley JH *et al.* Classification and grading of gastritis. The updated Sydney System. International workshop on the Histopathology of gastritis. *Am. J. Surg. Path.* 1996; **20**: 1161–81.

8 Haruma K, Mihara M, Okamoto E *et al.* Eradication of *Helicobacter pylori* increases gastric acidity in patients with