Acta Histochem. Cytochem. Vol. 29 No. 4 311-318 (1996)

Autoclave Antigen Retrieval Technique for Immunohistochemical Staining of Androgen Receptor in Formalin-Fixed Paraffin Sections of Human Prostate

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Received for publication October 8, 1996 and in revised form October 14, 1996 and re-revised form November 22, 1996

Although information on the states of androgen receptor (AR) expression at the individual cell level is essential in understanding the human diseased prostate, histological approaches are often hampered by the artifactual loss of AR or AR antigenicity. Here we examined the effects of various antigen retrieval methods, including microwave irradiation and autoclave treatment, for immunohistochemical detection of AR in cultured LNCaP cells and paraffin embedded sections of human prostate, and found beneficial effects of autoclave treatment over microwave treatment. Staining results were consistent with that of AR messenger RNA expression assessed by the reverse transcriptase-polymerase chain reaction method. The procedures described in this article provided intense and reproducible immunostaining for AR, estrogen receptor and progesterone receptor in paraffin sections of the human diseased prostate. Finally, 0.01 M EDTA (pH 7.4) gave us the most intense nuclear signal for the steroid hormone receptors, though the best soaking solution was 0.01 M citrate buffer (pH 6.0) considering the lost of tissue morphology.

Key words: Autoclave antigen retrieval, Immunohistochemistry, Androgen receptor, Steroid hormone receptor, Prostate

I. Introduction

In matter of fact, it has been well established that androgens play an important role in the development and function of normal prostate through androgen receptor (AR). The actions of androgens are also important to diseased prostate such as benign prostatic hyperplasia (BPH) or prostatic carcinoma. The expression and distribution of AR would be related to prognosis or alternativeness of therapy in prostatic carcinoma [23].

To investigate the correlation between AR contents and responsiveness to endocrine therapy in prostatic carcinoma, biochemical approaches including ligand binding assays were used [3]. However, it became apparent that those approaches were not enough to allow us to predict the effectiveness of hormonal therapy in individual patients, mainly because of contamination of non-malignant prostatic tissue in prostate cancer specimens [1]. Recently, both polyclonal and monoclonal antibodies against human AR have been introduced and applied to analyze AR expression of prostatic carcinoma at the level of individual cells [20]. Again, however, the relation between AR expression and response to therapy or progression after therapy has been controversial [11, 15]. It was suspected that AR extraction or a loss of antigenicity would occur during the conventional immunohistochemical procedures with routinely processed formalin-fixed, paraffin-embedded sections.

During the past decade, various antigen retrieval methods including microwave irradiation and autoclave treatment have been introduced to the immunohistochemical field and their beneficial effects were repeatedly documented [10, 12]. Shi *et al.* reported a microwave oven heating technique for retrieval of a variety of cell surface, cytoplasmic, and nuclear antigens [16]. Shin *et al.* developed a hydrated autoclave technique for the enhancement of tau protein immunostaining in formalin-paraffin sections [18].

In the present study, we examined several protocols of

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AR immunostaining with antigen retrieval methods such as microwaving and autoclaving in LNCaP cells as well as paraffin sections of human prostatic tissues, and compared the results of AR messenger RNA (mRNA) expression assessed by reverse transcription-polymerase chain reaction (RT-PCR). As a result, we found that the most intense and reproducible nuclear staining of AR was obtained with the autoclaving antigen retrieval procedure. The staining results were also verified by AR mRNA expression by RT-PCR. Moreover, we showed that the autoclave treatment was effective to the immunostaining of estrogen receptor (ER) and progesterone receptor (PR) in paraffin sections. The detailed protocol follows.

II. Materials and Methods

Cell lines and clinical specimens

LNCaP cells, which were derived from prostatic carcinoma metastasized to lymph node [6], were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 unit/ml penicillin, and 100 μg/ml streptomycin. The prostatic tissues analyzed in this study were three surgical specimens from patients with BPH, three surgical specimens from patients with clinically localized prostatic carcinoma undergoing radical prostatectomy, and three biopsy specimens from patients with prostatic carcinoma. The left supraclavicular lymph node to which prostatic carcinoma metastasized (Virchow's node) and lymph nodes without histologically detectable metastasis from patients with prostate cancer were also used.

Antibodies

Purified monoclonal antibody against AR protein (ARm, F39.4.1, a mouse IgG1 kappa), rabbit polyclonal antibody (ARp) against synthetic peptides corresponding to the part near the amino terminus that is specific to the AR, and purified monoclonal antibody against PR protein (PRm, 1A6, a mouse IgG1) were purchased from Novocastra Laboratories Ltd. (Newcastle, UK). Purified monoclonal antibody against ER (ERm, 1D5, a mouse IgG1 kappa) was purchased from Dako Co. (Glostrup, Denmark). Biotinylated rabbit anti-mouse IgG+IgA +IgM, biotinylated goat anti-rabbit IgG, normal rabbit serum, normal goat serum, normal mouse serum and an HRP-labeled streptavidin (SAB) kit were purchased from Nichirei Co. (Tokyo, Japan). Normal mouse IgG was purchased from Sigma Chemical Co. (St. Louis, USA).

Cell and tissue preparation

LNCaP cells cultured on plastic coverslip (Celldisk, Sumitomo Bakelite Co., Tokyo, Japan) were used for immunohistochemical study. After removal of the culture medium, coverslips were fixed in 10% formalin at 20°C for 10 min. The following procedures were carried out at room temperature (18°C–23°C) unless otherwise specified. The tissue specimens were immediately fixed with 10% formalin for 1–2 days after surgical removal or biopsy and embedded in paraffin in a routine manner. Five μm sections were cut, mounted on 3-aminopropyltriethoxysilane (Sigma Chemical Co.) coated glass slides [8], and heated in an oven at 42°C for 1 hr to promote adherence to the slide. After deparaffinization and rehydration, two types of antigen retrieval techniques were performed before reaction with antibodies.

Autoclave technique

The slides were placed in metal slide racks and immersed in a beaker filled with antigen retrieval solution. The beaker was loosely covered by a sheet of aluminum foil and then autoclaved at 120°C for 15 min (autoclave ASV-2402, Sakura, Tokyo, Japan). After cooling to room temperature, the lid of the autoclave equipment was taken off. In order to select an optimal antigen retrieval solution, the following buffers were tested for autoclave pretreatment; 0.01 M citrate buffer (Citrate) (pH 6), 0.01 M Citrate (pH 7.4), 0.05 M Tris-HCl (Tris) (pH 7.4), 0.05 M Tris (pH 8), 0.05 M sodium acetate (Acetate) (pH 6.0), 0.05 M Acetate (pH 7.8), 0.01 M phosphate-buffered saline (PBS) (pH 7.4), standard saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate) (pH 7.0), 0.01 M ethylenediaminetetraacetic acid (EDTA) (pH 6.0), 0.01 M EDTA (pH 7.4), 0.01 M Tris-HCl/ 0.001 M EDTA (TE) (pH 7.4) and distilled water (DW) alone.

Microwave technique

According to the protocol described by Shi *et al.* [16], microwave treatment was carried out. Briefly, the slides were placed in a plastic Coplin jar, which was filled with 0.01 M citrate buffer (pH 6.0). The slides were then irradiated in a domestic microwave oven (MR-6000AL, 600W, Hitachi, Tokyo, Japan) for 5 min at the maximal power. To compensate for evaporative fluid loss, the jar was refilled with the fresh buffer and the same procedure was repeated. After microwave irradiation, the slides were allowed to cool to room temperature.

Immunohistochemical staining of LNCaP cells and clinical specimens

Prior to immunohistochemistry, some slides of LNCaP cells were treated with autoclave and the others were left without any treatment. Tissue sections were treated with autoclave or microwave. The endogenous peroxidase activity was then blocked with 3% H_2O_2 in methanol for 15 min. Immunohistochemistry was performed using the monoclonal or polyclonal antibody and the signal was visualized by a modified avidin-biotinylated-peroxidase complex method, as detailed previously [5]. As a blocking solution, we used 10% normal rabbit serum for monoclonal antibodies and 10% normal goat serum for ARp, respectively. After blocking for 10 min, the sections were reacted with the primary antibody diluted at 1 : 10 for ARm, at 1 : 50 for ERm, at 1 : 40 for PRm or at 1 : 20 for ARp at 4°C for 12 hr in a moist chamber. After washing with 0.075% Brij 35 (Sigma Chemical Co) in PBS

three times, the sections were reacted with the appropriate prediluted biotinylated antibody (biotinylated rabbit anti-mouse IgG+IgA+IgM for monoclonal antibodies or biotinylated goat anti-rabbit IgG for ARp) for 10 min in a moist chamber. Then, the sections were washed and reacted with the prediluted HRP-labeled streptavidin for 5 min. After washing, the HRP site was visualized with DAB, H_2O_2, Co^{2+} and Ni^{2+} [8], without counterstaining. As a negative control, the specimens were reacted with normal mouse serum, normal mouse IgG, or normal rabbit serum in place of the specific antibodies after autoclaving or microwave irradiation.

Detection of AR mRNA by reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) for AR was performed to confirm the existence of AR mRNA in the cells and tissues used for AR immunostaining. The mRNA fraction was extracted as poly(A)RNA from the LNCaP cells and the clinical specimens by Quick Prep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden). An aliquot (0.1 μg) of each mRNA preparation was used for RT-PCR to detect AR mRNA and beta-actin mRNA. We synthesized complementary DNA (cDNA) from the isolated mRNA and amplified it by PCR according to the protocol described previously [4]. Two oligonucleotides used as PCR primers for detecting AR expression were devised from the published sequence of AR gene: AR-A, 5′-GCTATGAA-TGTCAGCCCATCT-3′(nucleotides No. 2153–2173, AR cDNA sequence); and AR-B, 5′-CAGAGGAGTAGTGC-AGAGTT-3′(nucleotides No. 2982–3001) [22]. The PCR with AR-specific primers produced a DNA fragment of 849 base pairs. The integrity of isolated mRNA was checked by RT-PCR with primers for human beta-actin as described previously [4]. Thirty cycles were performed, as follows: 60 sec denaturation step at 95°C, 30 sec annealing step at 55°C and 50 sec extension at 72°C. To minimize contamination, the sample preparations and the RT-PCR were performed by precautionary procedures as suggested by Kwok [9]. PCR products electrophoresed on 2% NuSieve 3 : 1 agarose gel (FMC BioProducts, Rockland, ME) were transferred onto Hybond-N+ (Amersham International plc, Buckinghamshire, UK) and analyzed by the Southern blot method [4]. An oligonucleotide, 5′-GCCC-CTGATCTGGTTTTCAA-3′(nucleotides 2449–2468) internal to the AR primers (AR-A and AR-B) was used as a probe for Southern blot analysis. The probe was labeled with fluorescein by an enhanced chemiluminescence 3′-oligolabeling system (Amersham) and allowed to be hybridized with the membrane. The hybridization signal on the membrane was detected by the enhanced chemiluminescence detection system (Amersham).

III. Results

As a model system, LNCaP cells were selected for assessment of effectiveness of autoclave antigen retrieval method using 0.01 M citrate buffer (pH 6). When LNCaP cells were autoclaved, strongly positive nuclear immunostaining of AR was observed using either polyclonal or monoclonal anti-AR antibodies. However, in formalin-fixed cells without autoclave pretreatment no nuclear immunostaining for AR was found under our experimental conditions (Fig. 1). In a negative control run with normal mouse IgG or normal rabbit serum, the specimens were essentially blank.

In order to confirm the synthesis of AR in LNCaP cells, the mRNA isolated from LNCaP prostate cancer cells was analyzed by RT-PCR with primers specific for AR gene. As shown in Figure 2, an 849-base pair DNA fragment was specifically amplified and Southern blot analysis further confirmed the identity of the fragment as a part of AR cDNA.

Prior to a wide application of autoclave antigen retrieval method to the paraffin-embedded tissue sections, we tested various soaking solutions used during autoclave for signal intensity and morphological preservation. As shown in Figure 3, we found the most intense signal for

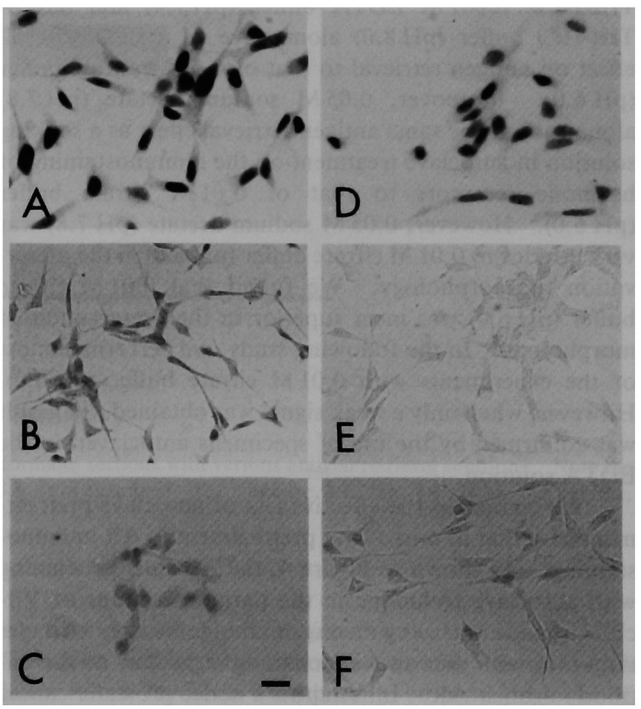

Fig. 1. The assessment of effectiveness of autoclave pretreatment in LNCaP cells. Polyclonal antibody (ARp) and monoclonal antibody (ARm) against androgen receptor were tested on LNCaP cells cultured on plastic coverslip. **(A)** autoclave (AC) + ARp; **(B)** AC+ normal rabbit serum; **(C)** no treatment (NT) + ARp; **(D)** AC+ ARm; **(E)** AC+ normal mouse IgG; **(F)** NT + ARm. Note strong staining in **A** and **D**, and negative staining in **B**, **C**, **E**, **F**. Bar = 10 μm (Original magnification × 100).

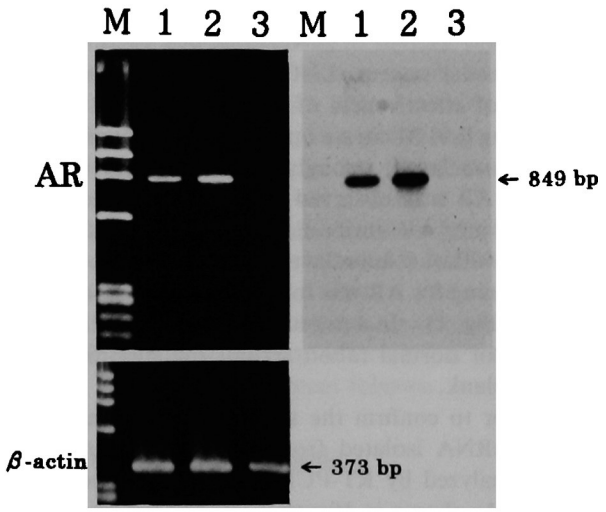

Fig. 2. Detection of mRNA from LNCaP cells (Lane 1), Virchow's node (Lane 2), and non-metastatic lymph node (Lane 3). RT-PCR was performed on each of these samples, using primers specific for androgen receptor mRNA or β-actin mRNA. Marker lane (M) was loaded with Hae Ⅲ-digested φ 174 DNA. bp: base pair.

AR with 0.01 M EDTA (pH 7.4) alone. However, 0.01 M EDTA (pH 7.4) was inferior to 0.01 M citrate buffer (pH 6.0) in the preservation of morphology. Both 0.01 M Tris-HCl/ 0.001 M EDTA buffer (pH 7.4) and 0.05 M Tris-HCl buffer (pH 8.0) alone gave us a slightly weak effect on antigen retrieval to that of 0.01 M citrate buffer (pH 6.0). Moreover, 0.05 M sodium acetate (pH 7.8) alone gave us the same antigen retrieval effect as a soaking solution in autoclave treatment on the immunostaining of hormone receptors to that of 0.01 M citrate buffer (pH 6.0). However, 0.05 M sodium acetate (pH 7.8) was very inferior to 0.01 M citrate buffer (pH 6.0) in the preservation of morphology. We found that 0.01 M citrate buffer (pH 6.0) was most superior in the preservation of morphology. In the following study, we performed most of the experiments with 0.01 M citrate buffer (pH 6.0). However, when only a weak signal was obtained, the result was confirmed by the use of specimens autoclaved in the EDTA solution.

We compared the effectiveness of autoclave pretreatment with that of microwave pretreatment in AR immunostaining. As shown in Figure 4, the AR nuclear staining with autoclave technique in the paraffin sections of Virchow's node was very strong, and significantly stronger than that with microwave technique regardless of the antibody against AR. In Virchow's node, prostatic cancer cells showed positive AR nuclear immunostaining, while lymphocytes and stromal cells showed no staining. Without any treatment, all cells showed no AR staining in Virchow's node. All cell types in the lymph node without metastasis showed no nuclear immunostaining using either polyclonal or monoclonal anti-AR antibodies, with autoclave pretreatment. In accordance with these findings, an 849-base pair DNA fragment of AR was amplified by RT-PCR in the cDNA preparation generated from Virchow's node, whereas the DNA fragment was not amplified in that from the lymph nodes without metastasis (Fig. 2).

With the protocol including autoclave pretreatment, we attempted to analyze AR, ER and PR expression in various diseased human prostates. In all 9 cases of prostatic tissue, a definite nuclear immunostaining of AR was found. In BPH specimens, the staining for AR was localized predominantly in the glandular epithelial nuclei as well as some of the basal cells. The stromal cells were also positive throughout the prostate. Most of the prostate cancer cells had moderate to strong nuclear immunostaining for AR using polyclonal and monoclonal antibodies specific for AR (Fig. 5). However, an intraregional heterogeneity in the AR immunostaining was observed both in the epithelial and stromal components of the cancers. The high intensity of AR signal and the low background staining in the slides treated with autoclave technique helped to distinguish positive cells and negative ones.

The staining for ER was frequently positive in periacinar stromal cell nuclei. The staining for PR was similar to ER immunostaining (Fig. 5). We found the most intense signal for PR and ER with 0.01 M EDTA solutions, though the solution was inferior to 0.01 M citrate buffer (pH 6.0) in the preservation of morphology. Negative controls for the slides after antigen retrieval pretreatment were incubated with either normal mouse serum or normal rabbit serum, showing a very low level of nonspecific staining in both the nucleus and cytoplasm.

IV. Discussion

The present study clearly demonstrated the usefulness of the autoclave antigen retrieval method in the immunohistochemical analysis of steroid hormone receptors such as AR, ER and PR in paraffin-embedded tissue sections. The results seemed beneficial because this approach permitted us to use the accumulated surgical specimens of human diseased prostate for AR expression analysis with the best performance. Moreover, the immunohistochemical results of AR were fully justified by RT-PCR analysis of the extracted mRNA.

In routine practice, most clinical human materials have been stored as 10% formalin-fixed, paraffin-embedded blocks throughout the world. However, often the immunoreactivity of a certain antigen with antibodies was reduced or lost in formalin-fixed, paraffin sections. AR is exactly such a case. Both polyclonal and monoclonal antibodies against AR used in our study gave no signals in the formalin-paraffin sections without pretreatment with the antigen retrieval techniques. Thus, it was a prerequisite for the immunohistochemical analysis of AR in paraffin sections that antigen retrieval methods were explored and optimized.

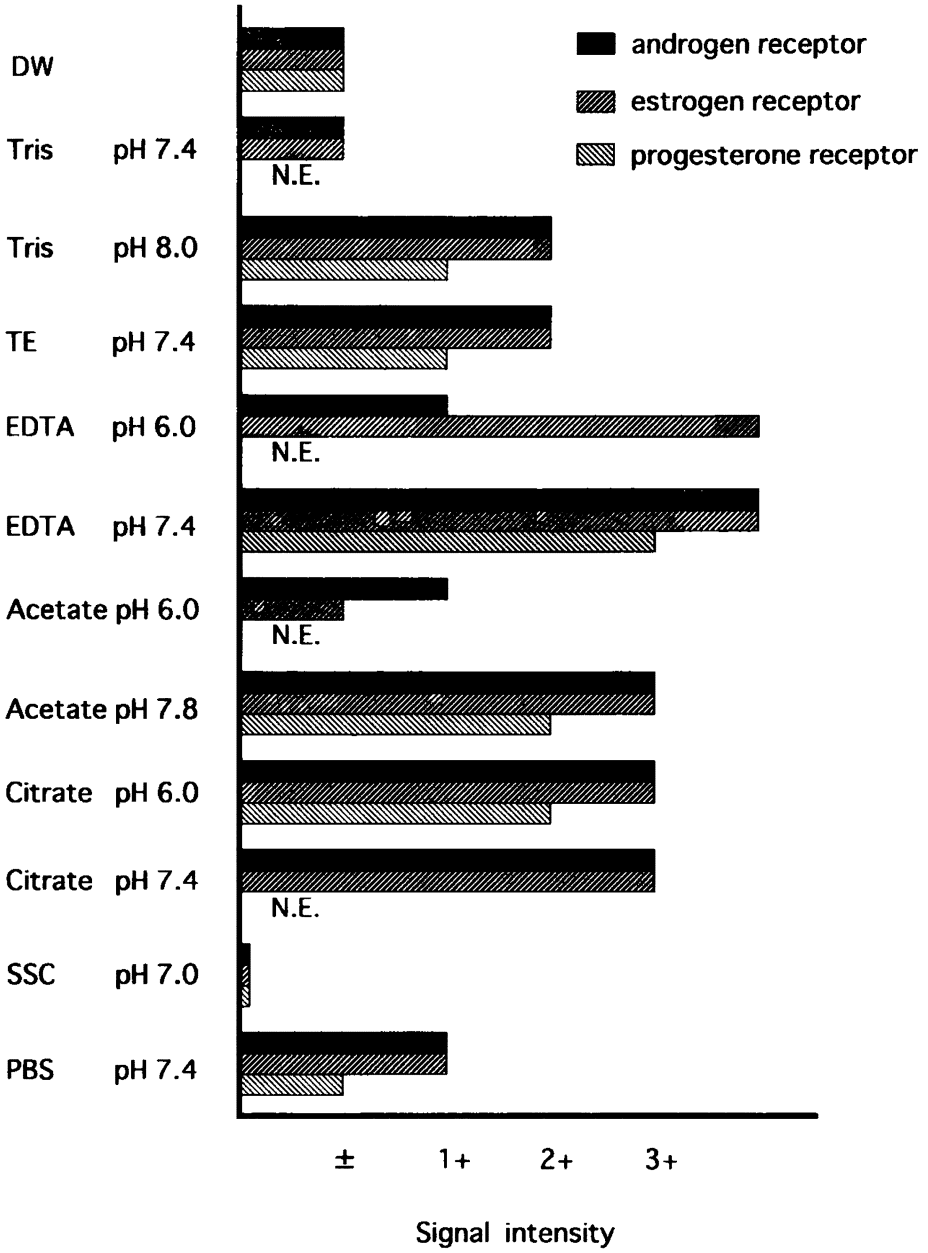

Fig. 3. Signal intensity of steroid hormone receptors immunostaining in formalin sections with various antigen retrieval solutions. Note the most intense signal for hormone receptors with 0.01 M EDTA pH 7.4. N.E.: not examined. (3+; intense, 2+; fair, 1+; weak, but definite, ±; faint)

Shi *et al.* reported the beneficial effect of microwave technique on AR immunohistochemistry in paraffin sections of prostatic tissues [16], suggesting that microwave heating may break the cross-links formed in proteins by formaldehyde [17, 21]. Janssen *et al.* also showed that the microwave technique was similarly effective to AR immunostaining in formalin-paraffin sections of other organs [7]. Besides the antigen unmasking effect, we have recently found a rapid, mild fixation of steroid hormone receptor by microwaving fresh frozen sections, resulting in an enhancement of immunohistochemical staining [19]. Therefore, we initially attempted to use microwave to detect AR in formalin fixed paraffin embedded blocks of prostatic tumors. However, the intensity of AR immunostaining in microwaved paraffin sections was still weaker than that of frozen sections [17]. Moreover, there were several cumbersome problems, including an optimization of temperature and duration of heating, a precise repetition of the constant heating process, and a limited number of slides treated at once [14, 21].

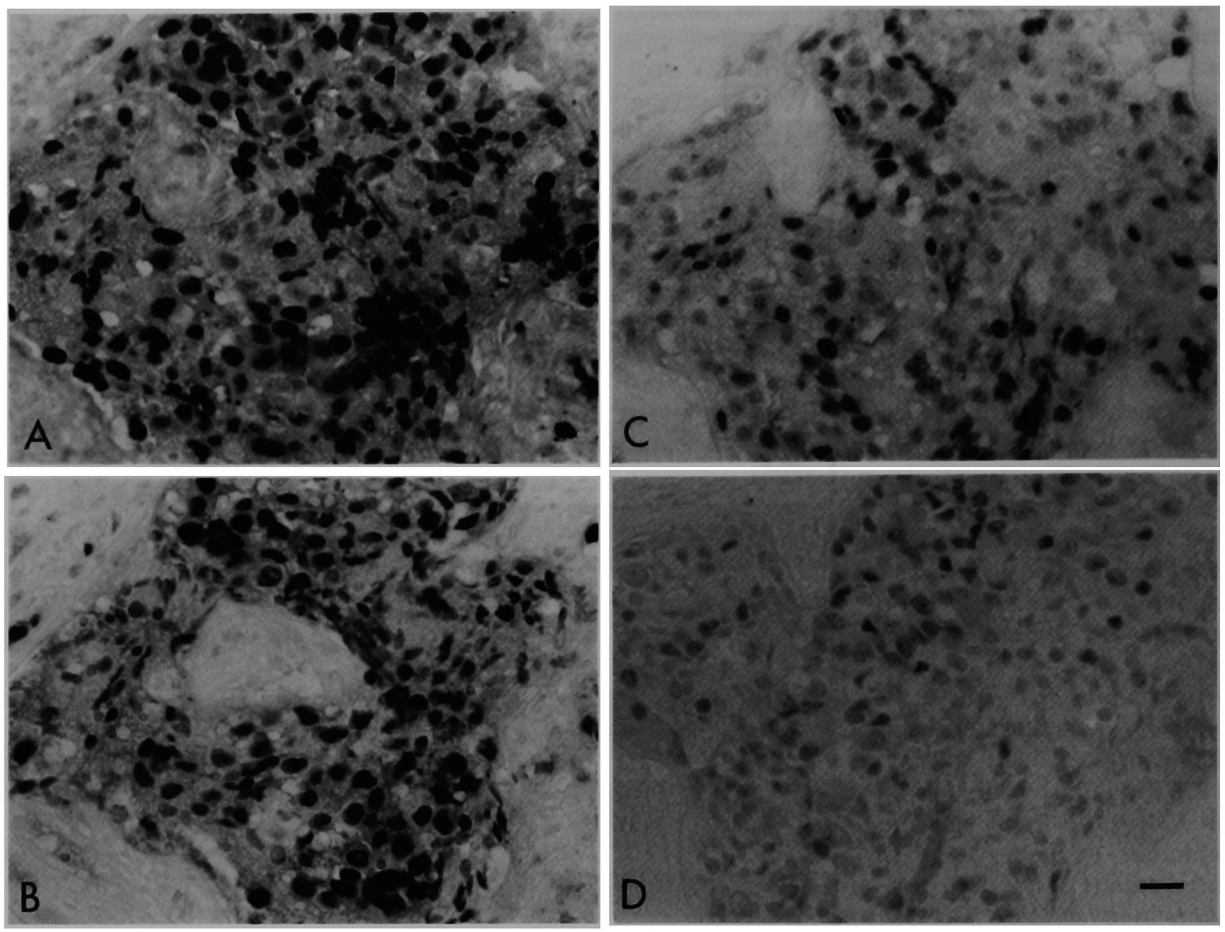

Fig. 4. Comparison of the immunohistochemical reactions using polyclonal antibody (ARp) and monoclonal antibody (ARm) against androgen receptor on formalin-paraffin sections of Virchow's node after autoclave (AC) and microwave (MW) pretreatment. **(A)** AC + ARp; **(B)** MW + ARp; **(C)** AC + ARm; **(D)** MW+ ARm. Note stronger nuclear immunostaining after AC than MW. Bar = 20 μm (Original magnification $\times$80).

Although we did not compare the AR immunostaining in paraffin sections with that in frozen tissue sections in this study, our results revealed that the AR immunostaining intensity with autoclave pretreatment was stronger than that with microwave pretreatment. Moreover, we confirmed various advantages in the use of autoclave pretreatment; the high reproducibility of results with a large number of slides, the ability to use metal slide racks, and the simplicity to standardize the treatment conditions [2, 14].

The mechanism of antigen retrieval by autoclave treatment still remains to be clarified. Norton *et al.* found that a domestic pressure cooker as a source of superheating was useful for the retrieval of a variety of cell surface, cytoplasmic, and nuclear antigens in formalin-paraffin sections [14]. Based upon their finding, it was suspected that superheating was the most critical factor of unmasking epitopes, which were once masked by fixation.

On the other hand, Taylor *et al.* showed that the pH value of the soaking solution is also a critical factor, in addition to superheating [21]. Recently, Bankfalvi *et al.* have reported that 0.01 M citrate buffer (pH 6.0), but not 0.05 M Tris-HCl buffer (pH 7.4), is useful as an antigen retrieval solution for immunohistochemistry with many antibodies including anti-ER and anti-PR [2].

In our hands, both 0.01 M Tris-HCl/0.001 M EDTA buffer (pH 7.4) and 0.05 M Tris-HCl buffer (pH 8.0) alone gave us a slightly weak nuclear signal and a very close morphological preservation effect in the immunostaining of steroid hormone receptors, compared to that of 0.01 M citrate buffer (pH 6.0). Interestingly, 0.01 M EDTA (pH 7.4) alone gave rise to the most intense nuclear signal for the steroid hormone receptors, though the preservation of tissue morphology was partially lost. However, DW had no beneficial effect. Moreover, increasing the salt concentration in soaking solutions resulted in only a deteriorative effect on both antigen retrieval and morphology. Considering that EDTA and citric acid act as a divalent cation chelator such as calcium, removal of some divalent cations may be important to unmask the cryptic

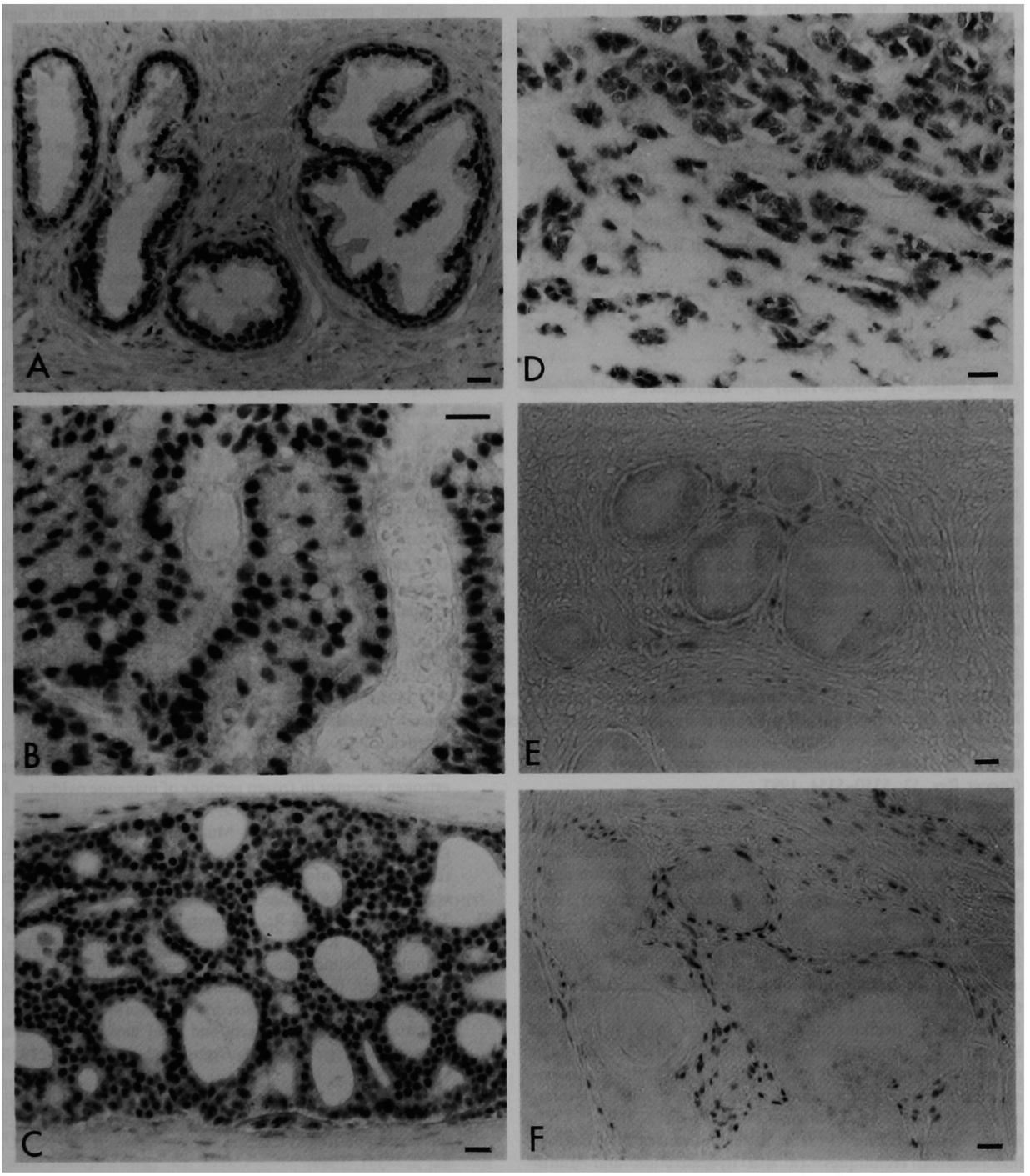

Fig. 5. Immunostaining of androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR) with autoclave pretreatment in formalin-paraffin sections of clinical specimens. **(A)** benign prostatic hyperplasia (BPH), using polyclonal anti-AR antibody (ARp); **(B)** well differentiated prostatic adenocarcinoma, using monoclonal anti-AR antibody (ARm); **(C)** moderately differentiated prostatic adenocarcinoma, using ARp; **(D)** poorly differentiated prostatic adenocarcinoma, using ARm, **(E)** BPH, using ERm, **(F)** BPH, using PRm. Bars = 20 μm (Original magnification: **A, C, E, F** ×50, **B** ×100, **D** ×66).

epitopes [13]. In the context, it should be noted that Tris has an amino group which can act as a possible recipient of the reactive aldehyde group released during the dissociation of cross-linking in proteins.

Finally, we obtained satisfactory results of AR immunostaining using an autoclave antigen retrieval technique in paraffin sections of clinical specimens. The AR expression in paraffin sections demonstrated the intraregional heterogeneity of prostatic carcinoma in accordance with the previous reports with frozen sections [11,

23]. In addition, the autoclave antigen retrieval method worked well on tissue sections, which were prepared 10 years, and the method will make it possible to establish a correlation between AR expression and prognosis, using surgical specimens from patients with a known clinical outcome.

We conclude that this autoclaving method of detecting AR, ER and PR in routinely processed formalin-fixed, paraffin-embedded sections of human prostate is highly useful, allowing retrospective studies on archival material on a large scale.

V. References

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