Preneoplastic and neoplastic cervical lesions as detected in Cytoblock® sections: The importance of sampling women with bleeding symptoms

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Summary

Purpose of investigation: The purpose was to use cervical samples to prepare Cytoblocks® and use the subsequent paraffin sections for additional immunostaining in our studies on angiogenesis.

Methods: Between January and April 2006, 261 women with bleeding complaints were selected of which 85 had gone to their general practitioner (GP) because of postcoital bleeding. The 261 cervical samples were processed by the Shandon Cytoblock® Preparation System. On the subsequent prepared Papanicolaou-stained paraffin sections a histological diagnosis was rendered on the minibiopsies.

Results: In all (pre)invasive cases, the paraffin sections contained numerous cancerous minibiopsies. The (pre)invasive cases had many Ki-67 positive nuclei displaying an S-phase staining pattern. In the Ki-67 stained sections, the glandular architecture of the two AIS cases and the two adenocarcinoma cases was highlighted.

*Conclusion:*Histologic paraffin sections provided enough minibiopsies to allow concise diagnosis including evaluation of proliferation. Signs of cervical angiogenesis, including postcoital bleeding, can be a strong argument to prepare cytoblocks from samples collected by sampling brushes.

Key words: Proliferation; Cervical angiogenesis; Cytoblocks.

Introduction

In the Netherlands, women are invited for cervical screening by their general practitioner (GP) when they turn 30, and every five years thereafter until age 60. The costs of these so-called invitation smears are paid by the health authorities. If screenees get symptoms in the years in between the screenings, they can go to their GP for interval cervical cytology.

In 2003 liquid-based cytology was introduced for cervical cytology, using the coagulant fixative BoonFix® (Finetec, Tokyo, Japan) as collection medium [1]. The method was first used for preparing PapSpin™ (Thermo Shandon) slides, with the already existing cytocentrifuge, the Thermo Shandon Cytospin. GPs were instructed to brush firmly, even till some bleeding occurred, to make sure that ample material was sampled. The sampling brushes were broken off so that the brush fit into the vial. In the laboratory, the vials containing the brushes were first placed in a shaker to remove collected minibiopsies and cells from the bristles of the brush. Only 10 to 20% of the sample collected was needed to prepare a ThinPrep slide: the rest of the material was viable to be embedded in paraffin for a cytoblock. The paraffin section contained many minibiopsies on which a histologic diagnosis could be made with ease [2]. In short, it was possible to render a histologic diagnosis based on material sampled by the GP with a Cervex-Brush® Combi (Rovers Medical Devices, Oss, The Netherlands). This finding inspired us to use cervical interval samples directly to prepare a cytoblock and to use this material to study the relationship between the histologic cytoblock diagnosis and clinical complaints related to angiogenesis. In addition, these paraffin blocks could be used to define proliferation patterns.

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Material and Methods

In total 261 consecutive cases were selected in which bleeding complaints were written on the data sheet and in which the vial contained a Cervex-Brush. The GP was informed that the total sample would be processed to a cytoblock.

Preparation of the cytoblocks

Cytoblocks can be prepared from samples collected for thin layer methods using the Shandon Cytoblock® Preparation System [3-5]. The vials still containing the brush were placed in a shaker for ten minutes to dislodge the material trapped between the bristles of the Cervex-Brush. Specimens fixed in BoonFix® were concentrated by centrifugation (10 min at 3000 rpm). The excess fluid was poured off from the pellet and a drop of 0.5% eosin was added. The cytofunnel and a cassette were placed in the cytospin clip. Two drops of the centrifuged sample were then poured in the Shandon Cytospin® cytofunnel (in which four drops of gelling solutions were added such that agar can surround and infiltrate the pellet). This was centrifuged for five minutes at 1500 rpm in the cytospin. The cytoblock cassette (containing the concentrated material infiltrated with agar) was removed from the cytospin and histoprocessed in the Pathos (Milestone, Italy) applying microwave technology. From each block several 4 mm sections were cut and stained with the Papanicolaou method.

Table 1. — *Benign diagnoses in Cytoblock® sections: bleeding symptoms.*

No.	Complaints	Normal	Endometrium	Micropolyps	Atrophia	Inflammation	Tissue repair	Total
1.	Postmenopausal bleeding	8	0	0	10	1	0	19
2.	Irregular bleeding	48	0	7	1	13	8	77
3.	Heavy bleeding	12	2	1	0	3	0	18
4.	Easily bleeding cervix*	20	0	4	2	3	1	30
5.	Spotting*	11	0	3	2	6	2	24
6.	Postcoital bleeding*	39	0	4	2	16	8	69
	Total	138	2	19	17	42	19	237

* very strong indication of cervical angiogenesis.

Table 2. — *(Pre)neoplastic cervical lesions detected in the Cytoblock®, sections: bleeding symptoms.*

No.	Complaints	No CIN	CIN I	CIN II	CIN III	AIS	SCC	Adenoca	Total
1.	Postmenopausal bleeding	19	0	0	0	0	0	0	19
2.	Irregular bleeding	77	1	0	0	0	0	0	78
3.	Heavy bleeding	18	1	0	0	0	0	1	20
4.	Easily bleeding cervix	30	2	0	1	1	0	0	34
5.	Spotting	24	1	0	0	0	0	0	25
6.	Postcoital bleeding	69	3	3	6	1	2	1	85
	Total	237	8	3	7	2	2	2	261

Ki-67 immunostaining

The Ki-67 (MiB-1) method was used to visualize proliferating nuclei in the minibiopsies. A microwave antigen retrieval step [6] was still needed, although shorter (5 min) than is needed for formalin-fixed tissues.

Results

Of the total 261 women screened, 237 had benign diagnoses, with only 138 having completely normal histology (Table 1). In 42 cases inflammation was suggested. The CIN cases (Table 2) contained minibiopsies with classical features of cervical neoplasia [7-9] which were discerned with ease in the excellent paraffin sections with optimal nuclear morphology. In the two cases of squamous cell carcinoma, many minibiopsies examined displayed squamous cells connected with extended desmosomes and hyperchromatic nuclei containing a macronucleolus (Figure 1).

In the Ki-67 stained sections, the nuclei were exclusively blue (hematoxylin, no Ki-67), or slightly blue and brown mainly located in and around the nucleolus (nucleolar Ki-67), or completely brown (the blue staining was overshadowed by the intense brown staining). The latter nuclei had a very coarse brown staining pattern occupying the complete nucleus.

Many benign cases had several endocervical minipolyps, with only an occasional Ki-67 positive reserve cell underneath the benign cylindrical epithelium (Figure 2). In three cases, the minibiopsies with tissue repair contained enlarged Ki-67 positive nuclei, the positive staining being mainly nucleolar (Figure 3). In the minibiopsies of CIN III, over 80% of the nuclei stained positive for Ki-67, with over 20% nuclei having an intense staining (Figure 4). The mitotic figures, when present, were also Ki-67 positive. Cases with CIN I and CIN II also contained Ki-67 positive nuclei, but in lower percentages. The two cases with SCC also contained many Ki-67 positive cells (Figure 5).

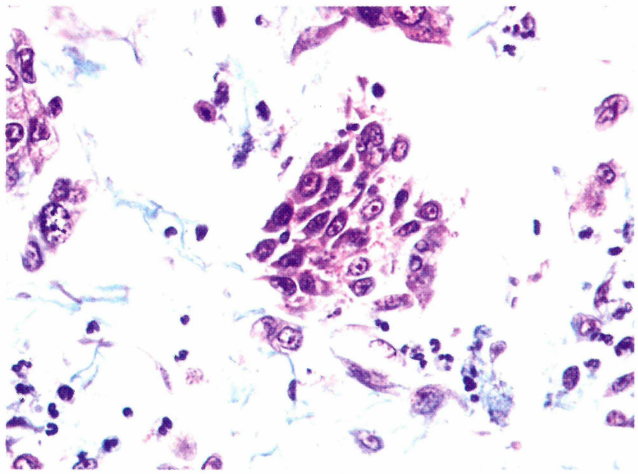
In one case of adenocarcinoma there were numerous carcinomatous fragments and almost no benign minibiopsies (Figures 6 and 7). In the Ki-67 staining, the glandular architecture was accentuated (Figure 8).

Discussion

Cervical sampling was changed fundamentally when in the eighties of the past century the cytobrush was introduced. Finally, the Ayre spatula was completely replaced by various brushes. Most immature lesions, including preneoplastic CINs, are often located in the endocervical canal and are effectively sampled by the brushes. Cancer detection was further improved by thin layer techniques suspending the cells. It has been reported that a histologic diagnosis can be rendered from material collected primarily for thin layer cytology, as a complementary technique [7-9]. To the best of our knowledge, this is the first study in which the cytology step was completely omitted. We decided on this approach for women sampled in between the five-year screening periods because we expected to catch many cases of CIN in these interval cases with overt bleeding complaints. Findings of 22 cases with (pre)invasive cervical carcinoma in 261 women validated our investigation.

When brushes such as the Cervex-Brush with stiff endocervical bristles are used, the endocervical lining containing CIN is vigorously sampled. In the past we saw that the bristles of the brush can act as "toothpicks" and effectively remove cancerous epithelium fragments from the endocervical clefts for inclusion in the sample. It has been our experience that not infrequently this com-

Fig. 1



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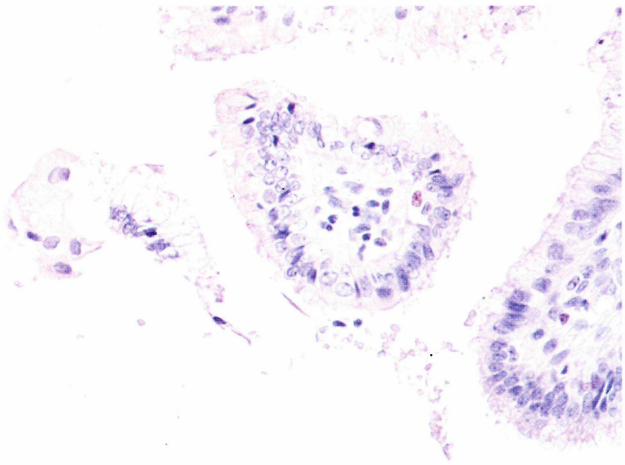
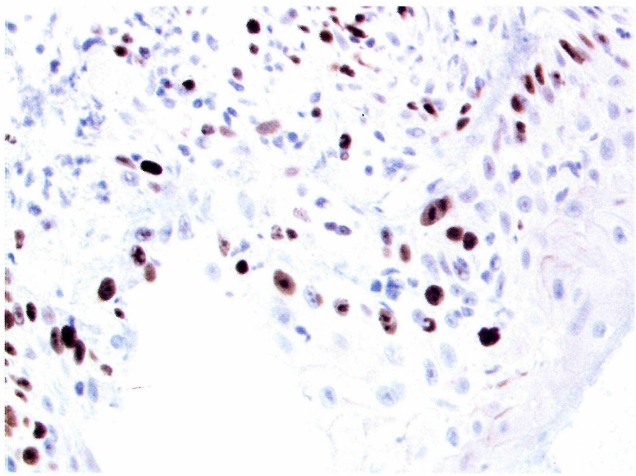


Fig. 3



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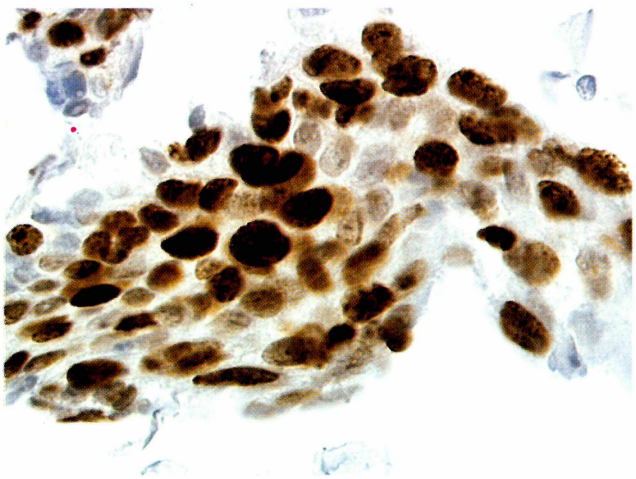

Figure 1. — Papanicolaou-stained paraffin cytoblock section. In this squamous cell carcinoma case many minibiopsies examined displayed squamous cells connected with extended desmosomes. The hyperchromatic nuclei contained a macronucleolus.
Figure 2. — Ki-67-stained paraffin cytoblock section. In this case the sections contained several endocervical minipolyps, with only an occasional Ki-67 positive reserve cell underneath the benign cylindrical epithelium.
Figure 3. — Ki-67-stained paraffin cytoblock section. In this case with tissue repair the minibiopsies contained enlarged Ki-67 positive nuclei, the positive staining being mainly nucleolar.
Figure 4. — Ki-67-stained paraffin cytoblock section. In this CIN III case the sections contained minibiopsies with over 80% positively staining nuclei. Note that there are nuclei with intense staining.

ponent is not included in the referred sample by simple washing of the brush in the suspension fluid because the epithelial fragments remain safely lodged in the mucus between the bristles of the brush. Accordingly, these important aspects of the sample along with the brush may end up in the clinician's or laboratory waste bin.

Our results indicate that inflammatory changes can lead to cervical bleeding, probably because inflammation is accompanied by angiogenesis. It is a well known fact that (pre)invasive cervical carcinoma is rich in newly formed small blood vessels [7], leading to postcoital bleeding [10-12].

The nuclear characteristics in the tissue sections were excellent allowing precise classification of Ki-67 in each nucleus. The (pre)invasive cases contained many Ki-67 positive nuclei displaying a S-phase staining pattern. Postcoital bleeding was present in six of the seven cases with CIN III and in both cases with SCC. Moreover, in three of the eight cases with CIN I and in all three CIN II cases there were very strong signs of postcoital bleeding, suggesting cervical angiogenesis.

Staining for proliferating cells can lead to subclassification of premalignant conditions in cytology and histology [13-15]. However, in cytologic slides there can be severe nuclear overlapping complicating the evaluation of proliferation. It is much easier to use histologic sections in which all nuclei are separated by the paraffin-impregnated cytoplasm and in which the nuclei are cut into thin slices [16, 17], making cytoblocks attractive for immunostaining [18].

Blood in cervical samples can be of clinical significance [19]. In this context it is important to mention that over 50% of the histologic sections contained blood. It is interesting to note that postcoital bleeding might be a sign of (pre)invasive carcinoma and it might be wise to use cytoblock technology in such cases.

Fig. 5

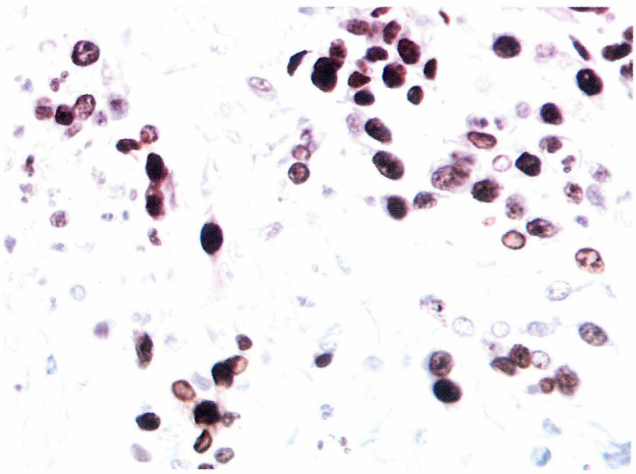

Fig. 6

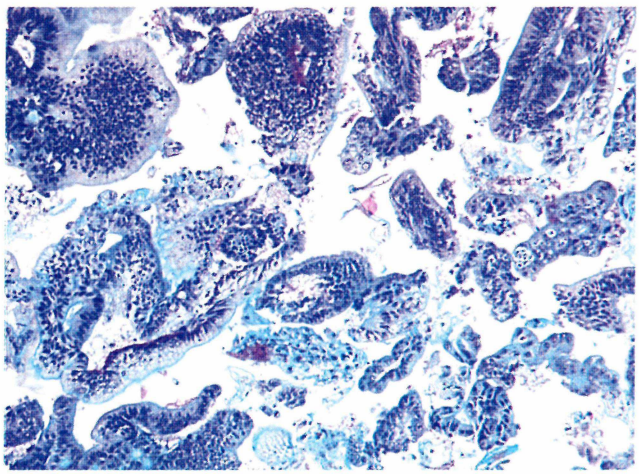

Fig. 7

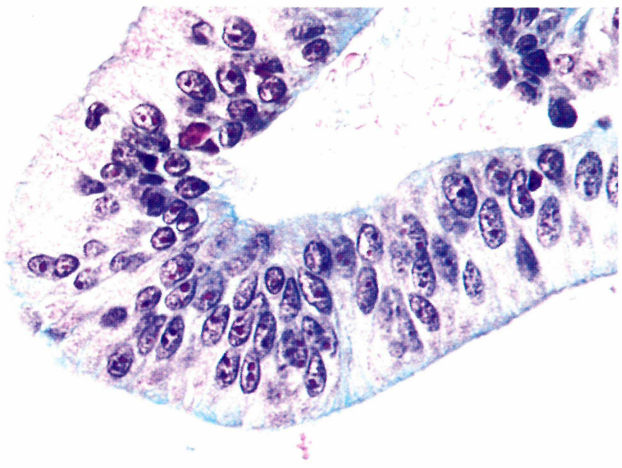

Fig. 8

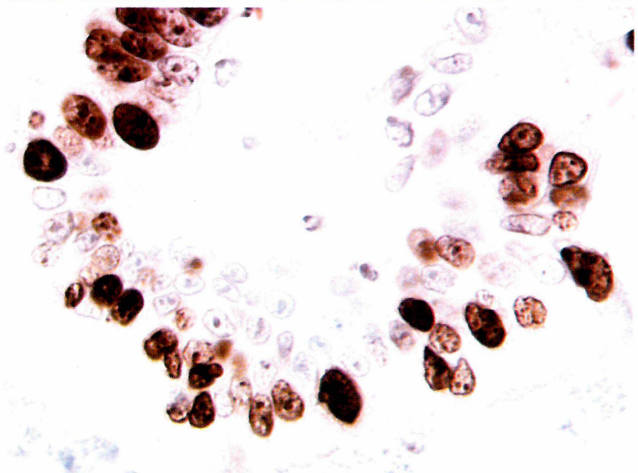

Figure 5. — Ki-67-stained paraffin cytoblock section. SCC with many Ki-67 positive cells. In this image many small minibiopsies can be seen.
Figure 6. — Adenocarcinoma with numerous large malignant fragments (Papanicolaou stain x 80).
Figure 7. — Same case as Figure 6. Every carcinoma cell nucleus can be studied in detail (Papanicolaou stain x 800).
Figure 8. — Same case as Figure 6. Proliferating cells are brown and many are completely stained. The glandular architecture is accentuated by the Ki-67 staining (Ki-67 immunostaining x 800).

Conclusion

Microscopy of cervical suspensions suspended in BoonFix and sampled with a Cervex-Brush® Combi can be optimized by paraffin embedding.

Of the 24 women with (pre)neoplastic cervical epithelium, 16 had a smear taken because of postcoital bleeding. It is advisable to prepare cell blocks of all women with postcoital bleeding and to educate women on the importance of this phenomenon.

References

[1] Boon M.E., Ouwerkerk-Noordam E., Suurmeijer A.J.H., Kok L.P.: "Diagnostic parameters in liquid-based cervical cytology using a coagulant suspension fixative". *Acta Cytol.*, 2005, *49,* 513.
[2] Boon M.E.: "Liquid-based versus conventional cervical cytology". Correspondence letter. *Lancet*, 2006, *367,* 1482.
[3] Yeoh G.P., Chan K.W.: "Cell block preparation on residual ThinPrep sample". *Diagn. Cytopathol.,* 1999, *21,* 427.
[4] Richard K., Dziura B., Hornish A.: "Cell block preparation as a diagnostic technique complementary to fluid-based monolayer cervicovaginal specimens". *Acta Cytol.,* 1999, *43*, 69.
[5] Rowe L.R., Marshall C.J., Bentz J.S.: "Cell block preparation as an adjunctive diagnostic technique in ThinPrep monolayer preparations: a case report". *Diagn. Cytopathol.,* 2001, *24,* 142.
[6] Suurmeijer A.J.H., Boon M.E.: "Pretreatment in a high pressure microwave processor for MIB-1 immunostaining of cytological smears and paraffin tissue sections to visualize the various phases of the mitotic cycle". *J. Histochem. Cytochem.,* 1999, *47*, 1015.
[7] Burghardt E., Ostor A.G.: "Site and origin of squamous cervical cancer: a histomorphologic study". *Obstet. Gynecol.,* 1983, *62,* 117.
[8] Johnson L., Easterday C., Gore H., Hertig A.: "The histogenesis of carcinoma in situ of the uterine cervix". *Cancer,* 1964, *17,* 213.
[9] Wright T.C., Ferenczy A.: "Anatomy and Histology of the cervix". In: Kurman R.J. (ed.). "Blaustein's Pathology of the Female Genital Tract", 5th edition, New York, Springer, 2001, 207.
[10] Emembolu J.O., Ekwempu C.C.: "Carcinoma of the cervix uteri in Zaria: etiological factors". *Int. J. Gynaecol. Obstet.,* 1988, *26,* 265.
[11] Rosenthal A.N., Panoskaltsis T., Smith T., Soutter W.P.: "The frequency of significant pathology in women attending a general gynaecological service for postcoital bleeding". *BJOG,* 2001, *108,* 103.
[12] Shalini R., Amita S., Neera M.A.: "How alarming is postcoital bleeding – a cytologic, colposcopic and histopathologic evaluation". *Gynecol. Obstet. Invest.,* 1998, *45*, 205.

[13] Boon M.E., Kleinschmidt-Guy E.D., Wijsman-Grootendorst A., Hoogeveen M.M.: "Upgrading unsatisfactory cervical smears with the MiB-1 method". *Diagn. Cytopathol.,* 1996, *15,* 270.

[14] Boon M.E., van der Veen G., Barlow Y., Graudenz M.S., Kok L.P.: "Presence of proliferating (MiB-1 positive) cells in cervical smears of women infected with HIV is associated with clinical outcome: a study of Brazilian women". *Diagn. Cytopathol.,* 2001, *24,* 373.

[15] Siemens F.C., van Haaften C., Kuijpers J.C., Helmerhorst T.J.M., Boon M.E.: "Progression of abnormal MiB-1 staining patterns of reserve cells in cervical smears from women ultimately developing high grade squamous intraepithelial lesions". *Acta Cytol.,* 2006, *50,* 637.

[16] Leteurtre E., Boman F., Farine M.O. *et al.*: "Importance of the study of the expression of MIB-1 (Ki-67) for the diagnosis of endocervical glandular lesions". *Ann. Pathol.,* 1998, *18,* 172.

[17] Payne S., Kernohan N.M., Walker F.: "Proliferation in the normal cervix and in pre-invasive cervical lesions". *J. Clin. Pathol.,* 1996, *49,* 667.

[18] Dagg B., Eustace D.L., Han X. *et al.*: "Cytoblock preparations for examination of cervical and other cells". *J. Clin. Pathol.,* 1992, *45,* 1122.

[19] Boon M.E., Ouwerkerk-Noordam E., van Leeuwen A.W.M.F. *et al.*: "Clinical and diagnostic significance of blood in cervical smears". *Diagn. Cytopathol.,* 2003, *28,* 181.

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