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Oestrogen Receptor Message in Premalignant and Normal Cervical Cells: A Methodological Study

Henry C. Kitchener, Lynn Neilson and Joan C.M. Macnab

Normal and abnormal biopsies of the uterine cervix, to a total of 124 samples, have been analysed for the detection of oestrogen receptor (ER) mRNA. The tough fibrous nature of the cervix proved very resistant to disaggregation in the first instance and subsequently, it was difficult to extract good high molecular weight message. This necessitated a systematic study of methodological technique, including two methods of tissue disaggregation and five techniques of extraction of ER mRNA, which in total involved the use of 124 cervical biopsies. The most successful method involved chopping the tissue, then digesting the cells with proteinase K and extracting the nucleic acids in salt and sodium dodecyl sulphate. Using the perfected technique, 16 cervical biopsies obtained at serial intervals from four women did not show any differences in ER mRNA in cervical biopsies either in the presence of oral contraception or histological abnormality. The successful method described will prove valuable for the detection of ER message in human tumours and other tissues of similar nature.
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INTRODUCTION

THE ASSOCIATION between human papillomavirus (HPV) and cervical cancer and precancer is well documented. Hybridisation studies using both Southern blot analysis [1] and polymerase chain reaction (PCR) [2] have indicated that there is a high rate of HPV16 detection in normal cervical tissues which suggests that cofactors other than HPV must influence cervical carcinogenesis. One such factor implicated in this context is smoking [3] which, as a putative promotor, could act on cervical cells in which abnormal cell changes had been initiated by viral transforming genes with the co-operation of activated oncogenes [4].

A hypothesis implicating oestrogen could be based on the following clinical observations. Cervical neoplasia generally arises in the so-called transformation zone (TZ) of the cervix which appears to be oestrogen-responsive. The TZ appears following the menarche, enlarges during the early reproductive era and pregnancy and then regresses postmenopausally. A source of exogenous oestrogen which could influence molecular events in an oestrogen responsive tissue is the combined oral contraceptive (OC), taken by millions of women of child-bearing age. In 1983, an epidemiological study appeared suggesting that long-term OC use conferred an increased risk of developing cervical cancer [5].

Previous reports from our laboratory showed that the level of transcription of the oestrogen receptor (ER) in oestrogen responsive cells could be increased, not only by exogenous oestrogen, but also by infection with herpes simplex virus (HSV). Moreover, the increase in the ER message in *in vitro* studies was shown to be induced by the viron trans-inducing factor (TIF) or Vmw65 [6], a component of the HSV virion. Exogenous oestrogen has been shown to be an obligatory requirement for the oncogenicity of MCF-7 cells in the nude mouse. In the absence of such oestrogenic stimulation, oncogenicity is only effected after transfection of MCF-7 cells with the oncogene V-*ras*^{H} [7].

The HSV TIF has also been shown to stimulate the early E7 ORF of HPV18 [8] whose expression is correlated with

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immortalisation of human keratinocytes. Thus, TIF activates a virus coded oncogene (E7) and the expression of a cell growth control factor, the oestrogen receptor.

To understand possible mechanisms associated with oncogenesis which could be operative in patients using oral contraception with or without concomitant virus infection, we investigated the levels of oestrogen receptor gene transcription in the cervix and correlated these with the histological appearance of the cervix.

MATERIALS AND METHODS

Colposcopically directed punch biopsies of the cervix were obtained from women attending the Glasgow Family Planning Clinic and immediately snap frozen in liquid nitrogen. All women gave informed consent. Some biopsies were from women with normal cytology who used various forms of contraception and were taken at different times during the menstrual cycle. Others were from women with abnormal cytology.

Extraction of the ER RNA from tissue biopsies could be divided into two stages: (i) the mechanical disruption of the tissues, and (ii) the extraction of good quality RNA from the disrupted tissue.

Both of these stages were extremely difficult to achieve. Two methods of tissue disruption were tested:

(a) Paired scalpels were used to chop tissue on a glass slide supported on frozen CO_2; success was dependent on dexterity and precision as well as a commitment to obtaining as much finely chopped tissue as speedily as possible. With repeated attempts it was possible to chop the tough fibrous cervical tissue finely.

(b) Different mechanical systems were tried but none was identified which succeeded in disrupting the very small biopsies invariably obtained. The systems tried were the Waring Blender, Microturrex Dounce Homogenizer and a mortar and pestle.

Extraction of RNA from the disrupted tissue involved an extensive comparison of five different systems. Briefly the methods of extraction were as follows:

Method 1 [9,10]. Chopped tissue was further dissociated in 4 mol/l guanidium isothiocyanate, 100 mmol/l 2-mercaptopurine, 0.5% w/v sarcosyl, 0.33% w/v antifoam A, 2 mmol/l EDTA and 50 mmol/l Tris–HCl pH 7.5. In this method, extracted nucleic acids were separated by caesium chloride centrifugation. The DNA was collected above a 5.7 mol/l cushion and the RNA was sedimented to the foot of the tube. Pelleted RNA was resuspended in 0.5% w/v SDS 5 mmol/l EDTA, 10 mmol/l Tris–HCl pH 7.4 and heated to 55°C for 1 min to dissolve.

Method 2 [11]. Chopped tissue was lysed in buffer (0.14 mmol/l NaCl, 2 mmol/l $MgCl_2$ 0.5% NP40, 10 μg/ml cycloheximide 0.2 Tris–HCl pH 8.5 for 5–15 min on ice. Nuclei and debris were removed by sedimentation (5 min, 3000 *g*, 4°C). The supernate cytoplasmic RNA was added to TBE (6.5% SDS, 5 mmol/l EDTA, 10 mmol/l Tris–HCl pH 8.5) and extracted thrice in phenol chloroform.

Method 3. Chopped tissue was suspended in buffer A (0.15 mol/l NaCl Tris–HCl, 1.5 mol/l MgCl, 0.65% NP40 pH 7.8, on ice for 3 min). Debris and cell nuclei were pelleted (3000 *g*, 10 min). Buffer B (7.0 mol/l urea, 0.35 mol/l NaCl, 0.01 mol/l EDTA, 0.01 mol/l Tris–HCl 1% (w/v) SDS pH 7.8) was added to the supernatant and RNA extracted thrice in phenol chloroform.

Method 4. 4 mol/l guanidium isothiocyanate was added to tissue, heated to 60°C and extracted in hot phenol (60°C) while the chromosomal DNA was sheared through an 18-gauge needle (adapted from [10]).

Method 5 [12]. Chopped tissue was added to nucleic acid extraction buffer (50 mmol/l Tris, 10 mmol/l EDTA, 100 mmol/l NaCl, 0.4% SDS pH 8.0 with 200 μg/ml proteinase K).

RESULTS

In total, 124 colposcopic biopsies of the cervix were obtained from 42 women. These were processed in an attempt to quantitate ER mRNA, but most resulted in degraded message until a successful technique was found. In order to evaluate the different methods, methods 1–5 were tested on 56, 12, 12, 8 and 36 biopsies, respectively. By far the most effective technique was method 5 which used finely chopped tissue and proteinase K to digest the cells with extraction of nucleic acids in salt and sodium dodecyl sulphate (SDS[4]). Chopped biopsy material was added to a standard nucleic acid extraction buffer of 50 mmol/l Tris, 10 mmol/l EDTA (TE), 100 mmol/l NaCl, 0.4% SDS pH 8 with 200 μg/ml proteinase K. Digestions were carried out usually for 0.5–2 h at 37°C but sometimes overnight for very resistant tissue and the sample either treated with DNAse (Promega) or the RNA separated by electrophoresis of total nucleic acids through an 0.8% agarose gel in TBE (0.089 mol/l Tris-borate, 0.089 mol/l boric acid, 2 mmol/l EDTA pH 7.8). Electrophoresis was carried out to separate the RNA [12] from the DNA and good separation was confirmed by viewing the gel under ultraviolet before the RNA was transferred to Hybond™ (Amersham International) and probed with a radiolabelled probe as a northern blot using the methods previously described [6].

After extraction, the RNA was quantitated either by running dilutions in a gel, visualising with ethidium bromide and comparing with known standards or by spectrophotometric analysis comparing with known standards and using the conversion 1 O.D. unit (260 nM) = 40 μg/ml RNA. Two different probes were used—(i) POR3; (ii) a synthetic oligonucleotide spanning nucleotides 258–373 (Exon 1, Pierre Chambon, personal communication) of the ER [13]. Probes were radiolabelled by end labelling, nick translation or primer extension. Mouse γ-actin, which is 85% homologous to human actin, was used as a standard to quantify the amount of RNA in each sample [6,12, 14]. It was established that amount of γ-actin did not depend on the time of the cycle the sample was taken (JCM Macnab, unpublished results).

The mechanical methods of disruption were all unsuccessful, either yielding small amounts of RNA (i.e. less than 8 μg per sample) or if a larger quantity (at best 15 μg per sample) was obtained, the integrity of the message was poor. Paired scalpels to chop the tissue on a slide supported on frozen CO_2 were more successful and thereafter biopsies were treated by this method.

The integrity of the ER message was checked on gels and most attempts at obtaining intact ER mRNA resulted in degraded message. The best results were obtained with the method described above, but successful extraction of ER mRNA was confined to only one group consisting of eight paired cervical biopsies (i.e. tissue from the TZ and NSE from the same cervix). These comprised serial samples from 4 patients with abnormal smears, 3 using OC and 1 intrauterine contraceptive device (IUCD) wearer. The duration of OC use was 9 months, 2 years and 11 years in the three OC users, respectively. The levels of ER message, together with the histology of the biopsies, is

Table 1. Correlation of ER message in eight pairs of cervical biopsies obtained from four women with histology of the transformation zone in comparison with internally paired native squamous epithelium

Specimen	Patient no.	Histology	Contraception	Ratio ER:actin	ER mRNA as % of control
1	34	Normal	OC 9 months	0.9	94
2	34	Int control	OC 9 months	0.62	95
3	52	Virus	IUCD	0.71	74
4	52	Int control	IUCD	1.28	134
5	52	CIN 1	IUCD	0.96	100
6	52	Int control	IUCD	0.90	94
7	52	Virus	IUCD	1.02	106
8	52	Int control	IUCD	1.19	124
9	Control	Cell RNA		0.96	100
10	Control	Cell RNA		1.22	100
11	38	CIN 3	OC 11 years	1.25	102
12	38	Int control	OC 11 years	1.21	99
13	38	Virus	OC 11 years	1.29	105
14	38	Int control	OC 11 years	1.01	83
15	2	CIN 3	OC 2 years	1.23	101
16	2	Int control	OC 2 years	1.29	105
17	2	Normal	OC 2 years	1.30	106
18	2	Int control	OC 2 years	1.07	87

In patients 52, 38 and 2 the biopsies were obtained serially. OC, oral contraceptive; IUCD, intrauterine contraceptive device.

shown in Table 1; the ER message level in each case being corrected with respect to the control, γ-actin. An example of the ER mRNA signal and γ-actin standard is shown in Fig. 1. The range for the ER mRNA from the TZ was 83–134 (mean 103), and the range for the NSE was very similar, 75–106 (mean 99). Correlation of ER with histological appearances showed no significant differences, and there was no correlation between ER and duration of OC use. The ER in the biopsies from the IUCD wearer was similar to the OC users.

DISCUSSION

Previous studies using oestrogen responsive cells in culture demonstrated that both exogenous oestrogen and virus common to the cervix, i.e. HSV, were capable of increasing the ER message [6]. Induction of ER by an exogenous supply of the hormone was essential to demonstrate the tumorigenicity of MCF-7 cells in the nude mouse unless these cells were transfected with the oncogene V-*ras*^{H} [7]. These results imply that the measurement of receptor message may be a useful means of monitoring whether exogenous oestrogen is exerting a functional molecular effect on the epithelial cells of the cervix.

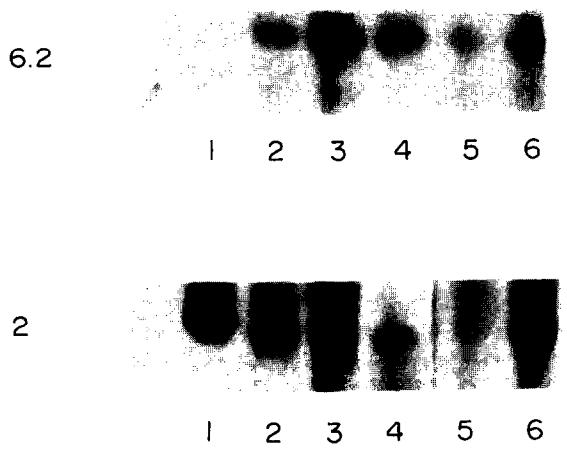

Fig. 1. Autoradiograph of a northern blot probed for the 6.2 Kb ER mRNA and the 2.0 Kb actin mRNA sequentially. Track 1 is the negative control for ER message and tracks 2–6, respectively, are cervical mRNAs from NTZ, NSE, NTZ, NSE and NTZ. (NSE, normal squamous epithelium; NTZ, normal epithelium from the transformation zone.)

A surprising feature of the study, however, was the difficulty experienced in obtaining good yields of RNA from cervical biopsies, and in extracting good quality high molecular weight ER message. The cervical biopsy proved very tough to chop and the method of collection, i.e. dropping into liquid nitrogen, seemed to aggravate the problems. The ER message, being of 6.2 Kb, was easily damaged by the extraction procedures. This problem had not been encountered when tissue culture cells were used [6]. It was essential to find a method of identifying the ER message which clearly showed its integrity and specificity of hybridisation to the probe. This meant that initially slot blot analysis was not practical as it was necessary to ensure that any hybridisation was to the intact message.

We reiterate that the only method which reproducibly gave good hybridisation to well-defined ER message was that of chopping the tissue and digesting this with proteinase K while extracting at 37°C in TE plus salt and SDS. Surprisingly, the message did not degrade even with prolonged extraction at 37°C. Good quality high MW ER mRNA hybridised to a radiolabelled ER probe and was quantified by subsequent hybridisation to a γ-actin probe. These samples were run on a gel and, once it was clear by visualisation under shortwave ultraviolet that the RNA was well separated from the DNA, RNA was denatured and transferred to the nitrocellulose [12].

Because of the technical difficulties encountered, most of the biopsies were expended in experiments concerned with methodology rather than the quantitative analyses, and our

results were confined to a small sample from which it is not possible to draw firm conclusions. Such preliminary results, however, suggest that the circulating levels of oestrogen in prolonged OC users do not result in the induction of raised levels of ER message nor does the latter appear to be the case in abnormal TZ compared with internal control tissue. The method of mRNA extraction may prove valuable for the detection of high molecular weight messages in other tissues refractile to extraction and ER message in other human tumours, as well as being a prototype study for ER message levels in cervix tissues.

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Interval Cancers and Sensitivity in the Screening Centres of the UK Trial of Early Detection of Breast Cancer

S.M. Moss, D.A. Coleman, R. Ellman, J. Chamberlain, A.P.M. Forrest, A.E. Kirkpatrick, B.A. Thomas and J.L. Price

The incidence rates of interval cancers following a negative breast screen in two screening centres which offered women aged 45–64 annual screening by mammography and/or clinical examination are examined. Sensitivity of screening is estimated by comparing the incidence rate of interval cancers with that expected in the absence of screening, and the results are compared with those from alternative methods of calculating sensitivity. The incidence rate of cancers diagnosed within 12 months of a negative screen by mammography plus clinical examination was reduced by 70% for women aged 45–54, and 84% for women aged 55+. There is no indication from this that sensitivity in the UK trial was substantially lower than in other studies which have achieved larger reductions in mortality.
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INTRODUCTION

A LARGE MULTICENTRE trial to assess the effect on mortality from breast cancer of screening by mammography and clinical examination, was started in the UK in 1979 [1]. After 10 years, an analysis of mortality showed a reduction in deaths from breast cancer of 20% in the two screening centres combined compared with four comparison centres ($P = 0.01$).

The rate at which new breast cancers appear following a negative screen is an important indicator of the sensitivity of the screening test, which will affect the potential benefit of screening. The increase in the rate of such interval cancers with time following a negative test also provides information on the natural history of the disease, and on the intervals at which screening should be performed to optimise the benefit:cost ratio. This paper gives information on interval cancers diagnosed in the two screening centres within the trial.