Seminal polyamines as agents of cervical carcinoma: Production of aneuploidy in squamous epithelium

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Abstract

The effects of several polyamines found in seminal fluid on the cell cycle and ploidy of three cervical cell lines and of primary epithelial cells cultured from cervical biopsy specimens were monitored by fluorescent flow cytometry. The rate of cell growth did not change but there were indications of either hypodiploidy or hyperdiploidy in some cultures at certain concentrations of spermine and spermidine. An interaction of exogenous polyamines with the DNA of cervical cells was shown to occur, leading to changes in ploidy with, perhaps, the potential to induce or promote dysplasia.

The cause of cervical squamous carcinoma is unlikely to be unique and may contain components which are both intrinsic and extrinsic to the cervix. The pubertal metamorphosis of the cervix provides the best known intrinsic component—the ectopy of columnar glandular epithelium with the emergence of a new type of squamous epithelium. The squamous replacement arises focally in the columnar and glandular ectopy by basal reserve cell proliferation and creates the transformation zone which is significantly prone to future malignancy. Malignancy in the transformational squamous epithelium, however, cannot be explained entirely by an intrinsic epithelial instability, and epidemiological and other evidence indicate that extrinsic events play a part. The epidemiological findings favour an agent transmitted sexually from male to female but are unable to specify its exact nature. Human papillomavirus (HPV) types, in particular, have been much studied and have established interesting and useful correlations with cervical neoplasia, but without defining a causal role. Less attention, however, has been paid to the possibility of transferable chemical agents acting either as carcinogens or as adjuvants to other agents.

The known growth promoting activities of plant and animal polyamines make them possible candidates. Seminal fluid contains several polyamines, particularly spermine, in concentrations (0.5–5.3 mg/ml) exceeding those used in demonstrating their effects in vitro. Polyamines are essential for cell growth, and raised concentrations of them and their limiting synthetic enzyme, ornithine decarboxylase, which produces the parent molecule putrescine, accompany periods of enhanced growth and differentiation in embryos and of regeneration in rat liver. The specific inhibitor of ornithine decarboxylase, 2-difluoromethyl ornithine, lowers polyamine concentrations and inhibits cell growth in vivo in animal tumours and in cell cultures of malignant cell type. The stimulation of cell growth and especially the prospect of an exogenous activation encouraged us to study the effects of the polyamines found in seminal fluid on cultured cervical squamous epithelium from the transitional zone and also on certain laboratory cell lines.

Methods

POLYAMINES
Spermine, spermidine, and 1,3-propanediamine were purchased from Sigma (Poole, Dorset). They were dissolved in 0.1 N HCl at a concentration of 1 mg per ml and diluted appropriately in medium just before use.

CELL LINES
SiHa and CaSkI cell lines were obtained from Dr H Cubie, and HeLa and Vero from Gibco Ltd (Paisley, Scotland). They were cultured as monolayers in Eagle's medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 200 μg/ml streptomycin and 5% pooled inactivated human serum. After trypsinisation the cells were subcultured into wells of tissue culture plates (Sterilin UK), 2 ml containing 1 × 10⁵ cells per ml in each well. The plates were incubated at 37 °C in 5% carbon dioxide in air, and one day later polyamines at various concentrations were added for up to 72 hours. Control cultures contained no polyamines.

CERVICAL CELL CULTURES
Cervical epithelium for culture came from normal (hysterectomy) cervixes within 30 minutes of removal from women between the ages of 27 and 50 years (mean = 41 years). Under the dissecting microscope the transformation zone was identified and the ring of predominantly squamous epithelium was incised around its inner and outer borders. Anterior and posterior sectors were then defined by two lateral radial incisions. The sectors were lifted by forceps and undercut by fine scissors as closely as possible to the epithelium. Samples for histological examination were taken to confirm their origin and absence of cervical intraepithelial neoplasia, HPV infection, or other abnor-
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Figure 1 DNA histograms of SiHa cultures after 48 hours of incubation: (A) control cells showing normal G0/G1 and its corresponding G2/M peak; (B) with 0.4 μg/ml spermidine showing an An (aneuploid) peak at aneuploid G0/G1 with a DNA index of 0.77 and a corresponding aneuploid G2/M peak with a DNA index of 1.55; (C) with 6 μg/ml spermidine showing aneuploid peaks with DNA indices of 0.78 and 1.65; (D) with 12 μg/ml spermidine showing aneuploid peaks with DNA indices of 0.76 and 1.60.

DNA ANALYSIS
At the end of the incubation period cultures were harvested by trypsinisation and further processed for DNA analysis by flow cytometry.
Figure 3 DNA histograms of Vero cultures: (A) control cells; (B) after incubation for 48 hours with 10 μg/ml spermidine showing a hyperdiploid peak at An G0/G1 with DNA index of 1.19.

Figure 4 Vero cells 48 hours after incubation with 10 μg/ml spermidine, stained with Rhodamine 123 (Sigma) at a concentration of 10 μg/ml. Excess stain was removed by washing the cells in 0.01 M phosphate buffered saline, pH 7.2. Cells were finally resuspended in medium containing 10% serum at 10^6 per ml and sorted on the basis of the log green fluorescence (LGFL) signal collected on a one parameter histogram. Gates were set to collect viable and non-viable cells at a flow rate of 1000 cells per second. Both populations of cells were then processed for DNA analysis as above.

Results

CELL LINES

HeLa, CaSki, and SiHa cell lines were chosen for this study as they were all derived from cervical carcinomas and contain HPV sequences. HeLa has 10-50 integrated copies of HPV-18 per cell with part of the genome from E2 to L2 missing. CaSki contains greater than 500 copies of HPV-16 per cell, all integrated, while SiHa has about 10 copies per cell of HPV-16, missing E2, E4, and some L sequences.

The three cell lines responded differently to the polyamines spermine and spermidine, used at concentrations of 0.4-50 μg/ml. In the case of SiHa cells no changes in DNA profile were seen at any concentration at 24 hours, but at 48 hours a hypodiploid peak was observed below the G0/G1 peak labelled An (aneuploid) (fig 1).
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Cervical cells after 15 days in vitro showing epithelial outgrowth from an explant.

The DNA index of this peak was shown to be less than 1.0 by incorporation of an external control (chicken red blood cells). There was also an equivalent hypertetraploid G2M peak. The paired hypoploidy occurred at all the polyamine concentrations, but by 72 hours the double peaks had disappeared and the only important change was in the percentage of cells in the G2M fraction of the cell cycle. This reduced from 10-2% in the control to a mean of 5-5% in cultures with added spermine and 1-2% with spermidine.

For CaSk-i cells, no changes were seen at 24 hours, but at 48 hours a hyperdiploid peak with a DNA index greater than 1.0 was observed in cultures receiving 5 µg/ml spermine or spermidine, or greater. These changes persisted at 72 hours. A similar picture was found for HeLa cells except the paired hyperploidy peaks were only seen at relatively high concentrations of polyamines (25-50 µg/ml). Analysis of the cell cycle compartments did not show any significant differences at the lower concentrations of polyamines. Examples of HeLa cells harvested at 48 hours are shown in fig 2.

The hyperploidy peaks were unexpected and further studies were done using Vero cells as these cells also showed a consistent hyperdiploid and hypertetraploid peak 48 hours after incubation with 10 µg/ml spermidine (fig 3A and B). The Vero cell line is fibroblastic and derived from the kidney of a normal African green monkey. It was decided to separate the cell population into viable and non-viable cells on the basis of uptake of rhodamine by the mitochondria of viable cells. Non-viable cells exhibit a weak, diffuse, green immunofluorescence. The cells were therefore stained and separated into non-viable and viable populations (fig 4A and B). The DNA stain was then carried out on each population. Figure 4D shows that the DNA from the viable cell population contained the hyperdiploid peak (seen as a "step" on the descending limb of G2/M), while the DNA from the non-viable cells did not (fig 4C).

Cervical Cells
Great care was taken to remove only the transformation zone of the hysterectomy specimens and 18 biopsy specimens were successfully cultured yielding squamous epithelial cell outgrowths, an example of which is shown in fig 5. The cultures were incubated in the presence of spermine, spermidine, or 1,3-propanediamine at concentrations ranging from 0.2-50 µg/ml and the cell cycle analysed. There was no significant difference within the cell cycle compartments, even at the highest polyamine concentration (table). On occasion, however, both spermine and spermidine at 5 µg/ml or higher gave rise to histograms which exhibited aneuploidy with a DNA index of less than 1-0 (fig 6). These samples have been excluded from the table. The aneuploidy was found in two samples with spermine at 5 µg/ml and four at 50 µg/ml, and in three samples with spermidine at 5 µg/ml and seven at 50 µg/ml. Only some cultures showing aneuploidy at 5 µg/ml were also aneuploid at 50 µg/ml. Aneuploidy was not seen with 1,3-propanediamine at any concentration.

Discussion
Most studies on polyamines have concentrated on the effects of their depletion in cells using inhibitors affecting their synthetic pathways. We wished to find out what happened to the DNA during the cell cycle of cervical cells when exogenous polyamines were present in the culture medium at or below the concentrations found in seminal fluid. The existence of a polyamine transport system has been shown in eukaryotic cells. Polyamines therefore accumulate intracellularly and are partially interconverted inside the cells.

Although the functions of polyamines in mammalian cells remain obscure, they seem to be essential for cell growth. The precise mechanisms of growth stimulation are difficult to define but include promoting or accelerating effects on all the major processes of prolifera-

<table>
<thead>
<tr>
<th>Polyamines</th>
<th>Number of samples suitable for parametric analysis</th>
<th>% of nuclei in G2/G1 phase (SD)</th>
<th>% of nuclei in S phase (SD)</th>
<th>% of nuclei in G2M phase (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18</td>
<td>81.8 (10.8)</td>
<td>12.4 (8.7)</td>
<td>5.4 (2.9)</td>
</tr>
<tr>
<td>5 µg/ml spermine</td>
<td>16</td>
<td>82.3 (7.1)</td>
<td>11.1 (4.6)</td>
<td>4.3 (1.9)</td>
</tr>
<tr>
<td>50 µg/ml spermine</td>
<td>11</td>
<td>79.1 (8.4)</td>
<td>15.7 (6.8)</td>
<td>4.8 (2.1)</td>
</tr>
<tr>
<td>5 µg/ml spermidine</td>
<td>11</td>
<td>78.0 (9.5)</td>
<td>17.7 (10.3)</td>
<td>4.6 (2.0)</td>
</tr>
<tr>
<td>50 µg/ml spermidine</td>
<td>8</td>
<td>76.5 (10.8)</td>
<td>18.1 (9.7)</td>
<td>4.4 (1.7)</td>
</tr>
<tr>
<td>5 µg/ml 1,3-propanediamine</td>
<td>16</td>
<td>83.0 (7.3)</td>
<td>12.3 (3.9)</td>
<td>3.9 (1.8)</td>
</tr>
<tr>
<td>50 µg/ml 1,3-propanediamine</td>
<td>16</td>
<td>88.5 (6.3)</td>
<td>13.1 (4.1)</td>
<td>6.1 (3.6)</td>
</tr>
</tbody>
</table>
Figure 6 DNA histograms of cervical cultures after incubation for six days with spermine at different concentrations: (A) control cells; (B) 5 µg/ml spermine; (C) 25 µg/ml spermine; (D) 50 µg/ml spermine; B, C, and D show hypodiploid peaks (An) with DNA indices of 0.71, 0.73, and 0.79, respectively.

Polyamines are known to change the conformation of DNA by affecting its condensation and by inducing its transition from a right-handed helix to a left-handed helix in suitable base sequences. Manning has proposed that the polyamines are able to neutralize the negatively charged phosphate groups on DNA and, in high concentration, could cause collapse, condensation, or compaction of the DNA molecule by reducing their mutual repulsion. Others think that the polyamines may occupy the minor groove of DNA rather than cover the phosphate groups and there is also some evidence for site binding and intermolecular cross-linking, all of which would influence the access of transcriptional enzymes and the action of regulatory sequences.

In this study we have shown that exogenous spermine and spermidine in some instances can affect the DNA of primary cervical cells and cell lines. There was no evidence that the polyamines, even at high concentrations (50 µg/ml), were cytoxic because the percentage of cells undergoing mitosis did not change.

Two unexpected effects, hypodiploidy and hyperdiploidy, were noted. These were not due to cell death as, in the case of hypodiploidy, an appropriate G2M peak was seen at a channel number twice that of the G0/G1 (figs 1 and 2), indicating that these cells are capable of division; in the case of hyperdiploidy cell sorting showed that the cells were viable. It is also possible that the polyamines had caused abnormal condensation of DNA in some cells, or perhaps had inhibited unwinding of the DNA helix, thus preventing the propidium iodide from intercalating and giving a false impression of the DNA content. Whatever the mechanism(s) it is clear that exogenous polyamines can change the structure of cellular DNA, and hence its function and regulation, and that they do so without loss of viability or mitotic potential. Thus the control of the cell may be sufficiently changed to predispose to dysplastic changes; further enhancement by other interacting factors, such as concurrent HPV infection, may be found. The protection afforded by cervical mucus is of obvious importance as it may prevent exogenous polyamines reaching the epithelial cell surface. On the other hand, high concentrations of nicotine have been found in mucus and may well act as adjuvants to prevailing concentrations of endogenous and seminal polyamines. Changes in cervical mucus with puberty, menstruation, and oral contraception may also influence the protection afforded.

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