Localization of proteasomes in human oocytes and preimplantation embryos

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In the present study we describe the localization of proteasomes in human oocytes, apoptotic preimplantation embryos, and triploid preimplantation embryos by means of immunolabelling with the MCP21 monoclonal antibody detected by confocal microscopy. While in the oocytes proteasomes are scattered throughout the cytoplasm, in the pronuclear zygote they appear to concentrate at the periphery of the cytoplasm and do not enter the pronuclei. During early cleavage stages, proteasome immunolabelling is concentrated in the nuclei, while the examination of triploid blastocysts showed that proteasomes had a similar cellular distribution to somatic cell lines, i.e. in the nuclei but not in the nucleoli or the cytoplasm. It appears that the distribution of proteasomes dramatically changes during human preimplantation embryo development.
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Introduction

The proteasome- and ubiquitin-dependent proteolytic system is responsible for the degradation of most proteins in the cells (Rock *et al.*, 1994). In order to be degraded by the 26S proteasomes, proteins must be first tagged with the polyubiquitin chain by the E1/E2/E3 cascade of enzymes (Varshavsky, 1997; Ciechanover and Schwartz, 1998; Tanaka, 1998). A notable exception to this rule is the degradation of ornithine decarboxylase, which instead of ubiquitination requires association with a specific co-factor called antizyme (Murakami *et al.*, 1999).

The 26S proteasomes are formed by 20S proteasomes associated with the PA700 activator (19S cap) (Coux *et al.*, 1996; Tanaka, 1998). The 20S proteasome is a barrel-shaped complex, formed by four stacked rings, two inner β rings and two outer α rings. These rings are composed of seven different subunits of the α and β family (Zwickl *et al.*, 1992; Coux *et al.*, 1996; Tanaka, 1998). The 20S proteasome has hydrolytic activities defined against small peptides as the chymotrypsin-like, trypsin-like, peptidylglutamylpeptide hydrolysing, small neutral amino acids preferring, and branched chain amino acid preferring activity (Orlowski, 1990). Free *N*-terminal threonine residues on three of seven β-type subunits are the active sites of this proteinase (Fenteany *et al.*, 1995; Seemuller *et al.*, 1995).

Proteasomes have been detected in all eukaryotic cells studied to date, as well as in the gametes of numerous species including starfish (Chiba *et al.*, 1997; Sawada *et al.*, 1999), ascidian (Kawahara and Yokosawa, 1992), fish (Tokumoto, 1999) and frog (Aizawa *et al.*, 1996) oocytes. In addition, their localization and possible function during embryonic development have been studied in several model organisms, including *Xenopus laevis* (Fujii *et al.*, 1991), *Pleurodeles waltl* (Pal *et al.*, 1988), *Drosophila melanogaster* (Klein *et al.*, 1990)

and chicken (Pal *et al.*, 1994; Hutson *et al.*, 1997). However, as far as we know, the localization of proteasomes in human or in any other mammalian oocytes and embryos was until now not investigated.

Proteasomes have been purified from mouse and human spermatozoa (Tipler *et al.*, 1997), where they are localized in the neck. Our unpublished observations suggest that in human spermatozoa the proteasomes apparently are not involved either in the acrosome reaction as in sea urchins (Matsumura and Aketa, 1991), or in sperm motility as in fish (Inaba *et al.*, 1993). In ascidians proteasomes take part in the fertilization process (Saitoh *et al.*, 1993), since proteasome inhibitors block it (Takizawa *et al.*, 1993); however, their role in mammalian fertilization is not understood. In lower animals they also perform specific proteolytic functions during the maturation of the oocytes (Chiba *et al.*, 1997; Sawada *et al.*, 1999) and metabolic activation of the zygote (Aizawa *et al.*, 1996).

In the present paper data are presented showing the localization of proteasomal antigens in human oocytes which failed to be fertilized *in vitro* and in abnormal human preimplantation embryos, which were either triploid or degenerated diploid.

Materials and methods

Collection of oocytes and embryos in a routine IVF procedure

Ovarian stimulation was carried out using a classical protocol associating desensitization with GnRH agonist in a long protocol, then stimulation by recombinant FSH. Both IVF and embryo transfer were performed using standard techniques as previously described (Segal and Casper, 1992). Mature, secondary oocytes were collected by transvaginal puncture of maturing Graafian follicles 36 h after the injection of 10 000 IU of HCG. After retrieval, oocytes from 28–38

year old women were cultured in IVF medium at 37°C in a humid atmosphere enriched in 5% CO_2. Insemination was carried out 3 h after oocyte retrieval with 1×10^5 motile spermatozoa/ml. At 18–22 h after insemination, oocytes were examined for the presence of two pronuclei. Those oocytes which contained the two pronuclei were further monitored and in case of normal development used for embryo transfer. Oocytes which failed to fertilize and which after a further 24 h culture were not cleaving were used for immunolabelling.

Developing embryos were graded into four categories (Dawson *et al.*, 1987). Grade I and II embryos were used for transfer and/or cryopreserved, while grade III and IV embryos (which are usually discarded because they exhibit a high degree of fragmentation and generally arrest at early stages of development) were used for immunolabelling. Also embryos developing from zygotes with three pronuclei (triploids) were used for the observations.
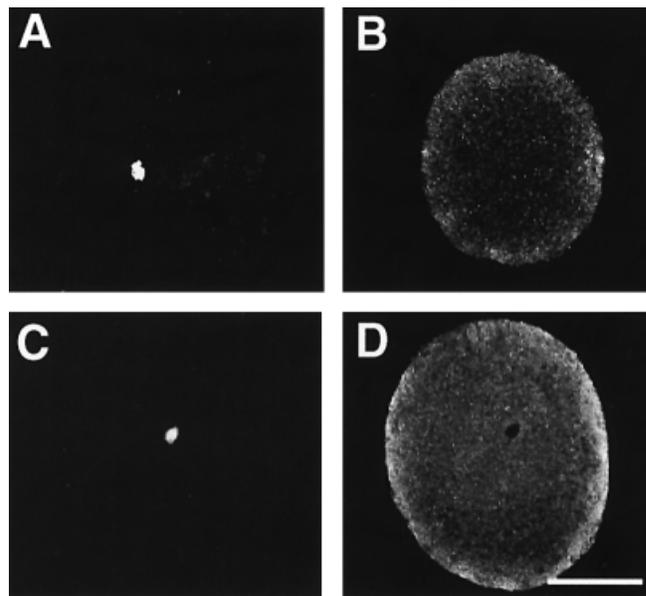
Immunolabelling

Oocytes and embryos were transferred from and into the different solutions by the use of a mouth-controlled pipette. After washing in phosphate-buffered saline (PBS), zonae pellucidae were removed by a 10 min immersion in acid Tyrode's solution. Immediately afterwards they were fixed in 2% formaldehyde for 1 h, then quenched in 50 mmol/l NH_4Cl in PBS, permeabilized in 0.1% Triton X-100 in PBS, washed once in PBS and twice in Tris-buffered saline (TBS, pH 7.6) supplemented with 1% bovine serum albumin (BSA) and 1% fish skin gelatin. Incubation with MCP21 monoclonal antibody (kind gift of Dr. Klavs Hendil, August Krogh Institute, University of Copenhagen, Copenhagen, Denmark) (Kaltoft *et al.*, 1992) diluted 1:100 in TBS supplemented with 0.1% Tween-20, 1% BSA and 1% fish skin gelatin was carried out in a dark humid chamber for 2 h. After triple washing in TBS supplemented with 1% BSA and 1% fish skin gelatin, they were incubated with Cy^{TM} 3-conjugated donkey anti-mouse AffiniPure immunoglobulin G (IgG) (H+L) (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:1000 in TBS supplemented with 0.1% Tween-20, 1% BSA and 1% fish skin gelatin. After washing in TBS, oocytes and embryos were treated with RNA-se A (10 mg/ml in 0.01 mol/l sodium acetate, pH 5.2) for 1 h at 37°C and counterstained for 10 min with Yo-Pro™-1-iodide (Molecular Probes, Eugene, OR, USA) dissolved in TBS at a concentration of 1 µmol/l. Finally, the oocytes and embryos were placed carefully on microscope slides, embedded in fluorescence mounting medium (Dako Corporation, Carpinteria, CA, USA) and coverslipped.

As a control for the specificity of the MCP21 antibody, three oocytes were incubated with a non-immune mouse serum instead of the primary antibody. As a control for the secondary antibody, four oocytes were incubated without the primary antibody. We did not perform any preadsorbtion/blocking protocols, because of the high specificity of the MCP21 antibody, which detects the HC3 α-type proteasome subunit both in purified proteasome preparations and in cell lysates (Kaltoft *et al.*, 1992; K.Hendil, personal communication; Wojcik *et al.*, 1995b).

Fluorescence confocal microscopy

After mounting, samples were kept for up to 1 week at 4°C in darkness before examination with an LSM 10 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The excitation source was a 488 nm wavelength argon laser, and fluorescence emission was recorded by two detectors situated behind a 590 nm long pass, a filter and a 515–545 nm band pass. Both excitation and emission light were focused through a ×40 Plan-Neofluar immersion objective with 1.3 numerical aperture and an additional electronic zoom produced

Figure 1. Two unfertilized oocytes arrested at the germinal vesicle stage observed by confocal laser scanning microscopy. (**A**, **C**) DNA labelled with YoPro™-1-iodide and (**B**, **D**) the same optical sections immunolabelled with MCP21. Bar = 25 µm.

the definitive images. Optical sections were acquired, each one with an image resolution of 512×512 pixels.

Results

Observations of three oocytes incubated with a non-immune mouse serum and four oocytes incubated without any primary antibody revealed no immunolabelling. The faint background fluorescence was too weak to be collected by confocal microscopy.

Observations of nine germinal vesicle stage arrested oocytes showed that, in all cases, MCP21 immunolabelling was present uniformly throughout the cytoplasm, however the germinal vesicle chromatin was devoid of labelling. Cytoplasm immunolabelling displayed a fine granular structure, since apparent negative shadows of presumptive vacuolar structures were also always detected. The immunolabelling at the oocyte cortex appeared to be enhanced, compared with the central ooplasm which showed a mild gradient (Figure 1).

Observations of 13 oocytes which had arrested at metaphase II showed basically the same pattern. Proteasomes appeared to be distributed uniformly throughout the cytoplasm with an enhancement at the cortex, while the metaphase plate was devoid of labelling (Figure 2A,B). In polar bodies, chromatin was devoid of labelling and proteasomes concentrated at the periphery (Figure 2C,D). In two cases a slight enhancement of the immunolabelling was seen in the surroundings of the metaphase plate of the oocyte, most probably corresponding to the localization of the spindle (Figure 2E,F).

Four otherwise normal zygotes, which arrested at the pronuclear stage despite prolonged cultures were also observed. Three of them showed strong peripheral MCP21 immunolabelling (Figure 3A,B), while one of them showed a more diffused pattern (Figure 3C,D). However, none of them appeared to
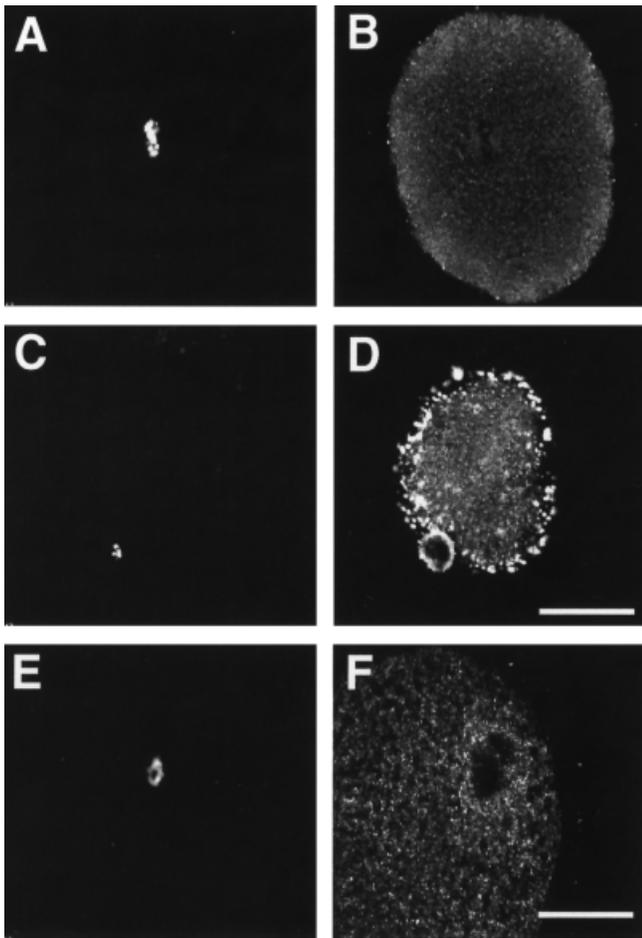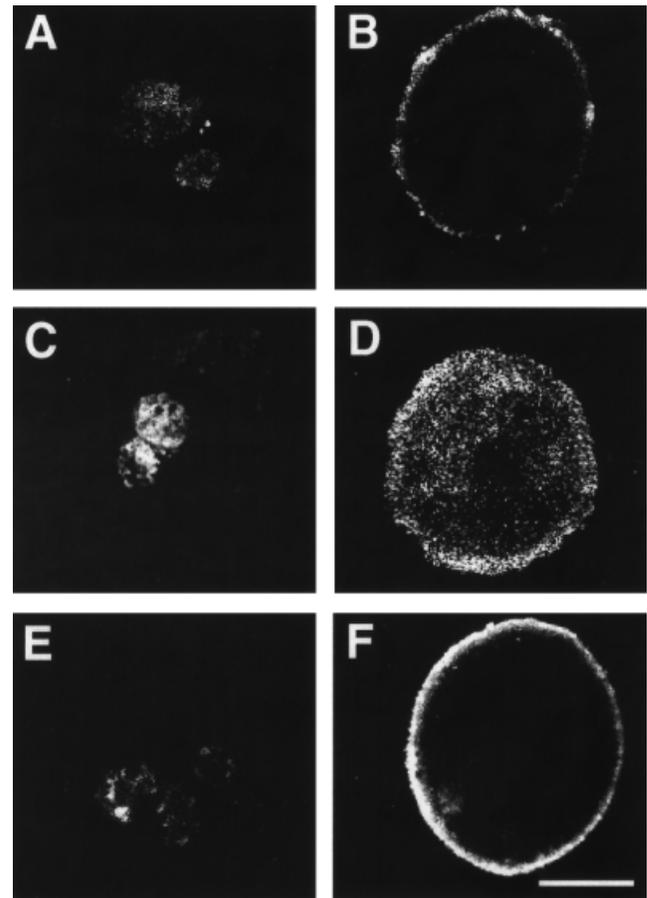
Figure 2. Two unfertilized oocytes arrested at metaphase II stage observed by confocal laser scanning microscope. (**A**, **C**, **E**) DNA labelled with YoPro™-1-iodide and (**B**, **D**, **F**) the same optical sections immunolabelled with MCP21. (**A**) Metaphase plate of one oocyte is seen, while on a different optical section (**C**, **D**) the chromatin of the polar body is seen. Bar = 25 µm. (**E**) The metaphase plate of a different oocyte is shown at a higher magnification. (**F**) MCP21 immunolabelling is slightly enhanced at the presumptive location of the spindle. Bar = 10 µm.


Figure 3. Three arrested pronuclear stage zygotes which failed to cleave after prolonged culture, observed by confocal laser scanning microscope. (**A**, **C**, **E**) DNA labelled with YoPro™-1-iodide and (**B**, **D**, **F**) the same optical sections immunolabelled with MCP21. While the first two zygotes have only two pronuclei (**A**, **B**, **C**, **D**), the third one is triploid (**E**, **F**). N.B. in all cases pronuclei are devoid of MCP21 immunolabelling. Bar = 25 µm.

have any proteasomes inside the pronuclei. Moreover, an identical pattern was seen in two triploid zygotes (Figure 3E,F).

A total of 24 embryos of further stages of development, which arrested after culture *in vitro* and/or were discarded due to their poor morphology, all displayed different degrees of apoptosis, as judged by chromatin condensation in their nuclei (Figure 4). Strikingly, in most cases, the MCP21 immunolabelling was concentrated inside the nuclei (Figure 4A,B). While in some cases, proteasomes and DNA were co-localized, in other cases they were not and, therefore, were concentrated in different domains of the apoptotic nucleus. In two cases of very advanced embryo fragmentation with chromatin scattering throughout the cytoplasm and no recognizable nuclei, the proteasomes showed a peripheral cytoplasmic localization (Figure 4G,H).

For ethical reasons, it was impossible to perform similar observations in normal human embryos. However, the use of a fragmented triploid 3-cell embryo and a triploid blastocyst allowed some observation. Figure 5A,B shows an isolated

blastomere from a triploid 5-cell embryo with apparently normal chromatin morphology. The proteasomes are located inside the nucleus and at the periphery of the cell. In a triploid blastocyst, the MCP21 immunolabelling is similar to that seen in cultured cell lines (Figure 5C,D). The proteasomes are mainly nuclear in interphase cells, but some labelling is also cytoplasmic, as it can be seen at higher magnification (Figure 5E,F). No differences were noted between the inner cell mass and the trophectoderm.

Discussion

The MCP21 monoclonal antibody specifically recognizes the HC3 α-type proteasome subunit (Kaltoft *et al*., 1992; K.Hendil, personal communication). It detects a single band in lysates of human spermatozoa and in purified proteasome preparations (unpublished observations), while in HeLa cells it detects two bands of similar molecular size most probably corresponding to isoforms of the same subunit (Wójcik *et al*., 1995b). With the exception of the β-type subunit zeta, no proteasome subunits are known to exist in the cells as free proteins, not associated with the proteasome complex (Jorgensen and Hendil,
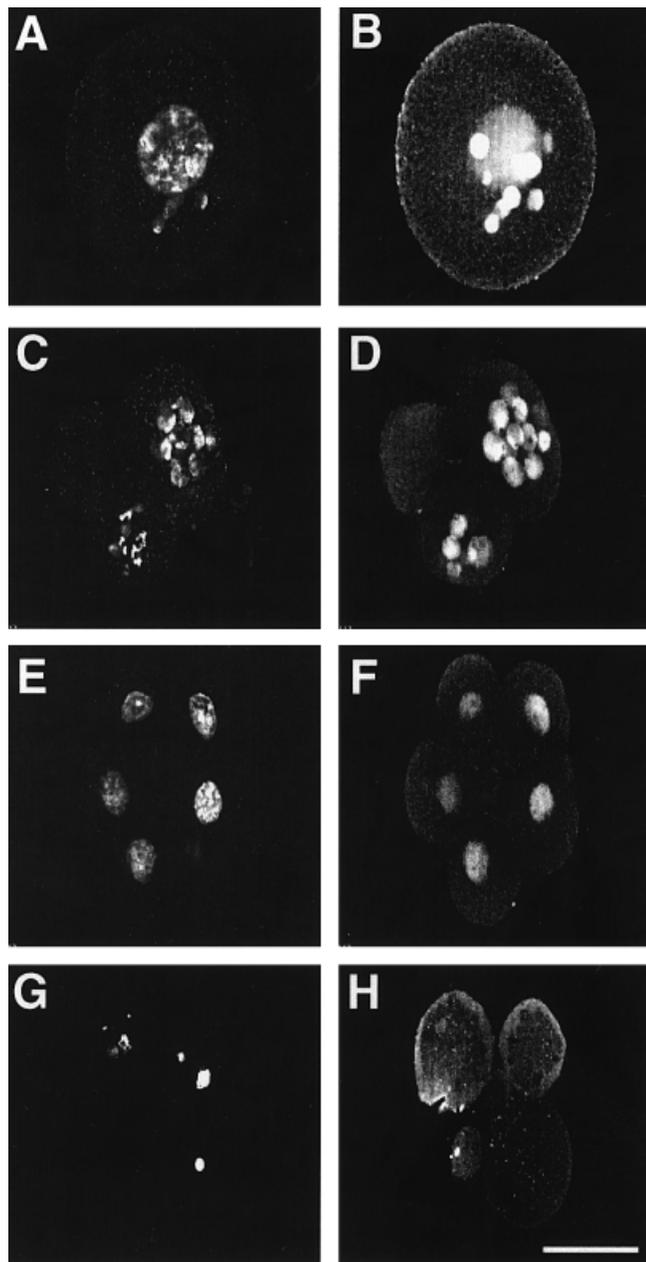
Figure 4. Four apoptotic embryos arrested at different stages of development displaying different abnormalities observed by confocal laser scanning microscope. DNA labelled with YoPro™-1-iodide (**A**, **C**, **E**, **G**) and the same optical sections immunolabelled with MCP21 (**B**, **D**, **F**, H). An embryo which probably failed to cleave after syngamy (**A**, **B**), a highly multinucleated two-cell embryo (**C**, **D**), a 7-cell embryo (**E**, **F**) and a fragmented embryo with advanced chromatin degradation (**G**, **H**). Bar = 25 µm.

1999). Therefore, we considered that immunolabelling with the MCP21 antibody reflects the localization of intact 20S proteasomes (either alone or associated with PA700 activator to form 26S proteasomes). A small fraction of the immunolabelling can also correspond to the localization of the pre-assembly 20S proteasomes.

Due to ethical reasons, only oocytes which failed to be fertilized and embryos which were considered abnormal, either due to triploidy or due to different degrees of morphological alterations and abnormal rate of development, were used. On
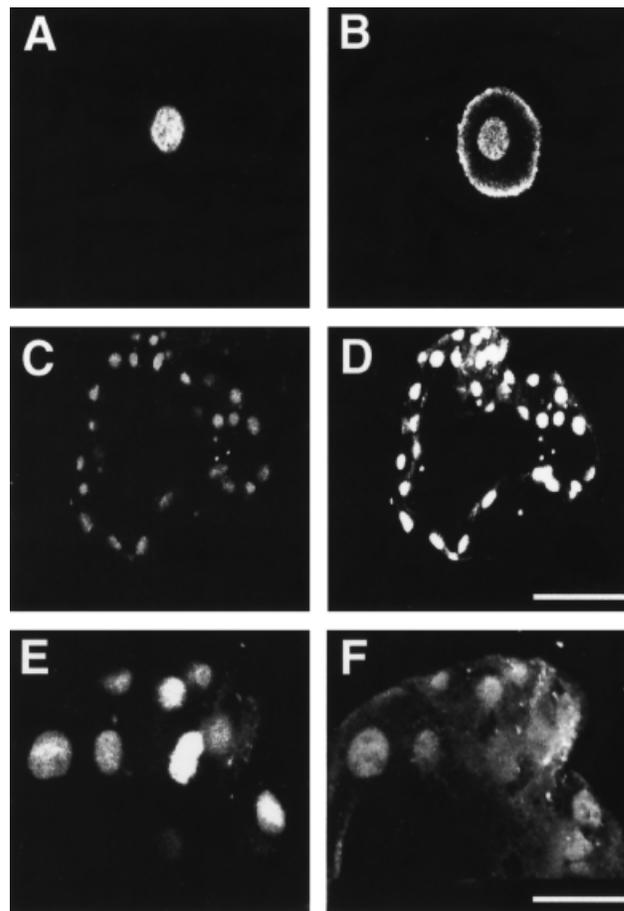
Figure 5. A single triploid blastomere from a fragmented 5-cell stage embryo (**A**, **B**) and a triploid blastocyt observed at (**C**, **D**) low or (**E**, **F**) high magnification, observed by confocal laser scanning microscope. DNA labelled with YoPro™-1-iodide (**A**, **C**, **E**) and the same optical sections immunolabelled with MCP21 (**B**, **D**, **F**). (**A**–**D**) bar = 25 µm; (**E**, **F**) bar = 10 µm.

the basis of our observations, we infer that proteasomes show abrupt changes in their localization during oogenesis, fertilization and further development.

In somatic cells, proteasomes are present in the nuclei with the exception of the nucleoli, and in the cytoplasm (Rivett *et al*., 1992; Amsterdam *et al*., 1993; Wojcik *et al*., 1995b). During mitosis, they are not associated with the chromosomes, however they usually concentrate at the spindle apparatus (Amsterdam *et al*., 1993; Wojcik *et al*., 1995b). This is consistent with our observations of unfertilized oocytes, since proteasomes are present in the ooplasm but are not associated with the chromatin, even at the germinal vesicle stage, which corresponds to the first meiotic prophase. In two cases we have found an increased immunolabelling in the region of ooplasm surrounding the metaphase plate, which could represent proteasomes associated with the spindle apparatus. In other cases we were not able to find such an increased immunolabelling, however it is important to keep in mind that these metaphase plates were often abnormally arranged, with scattered chromosomes, which could represent a spindle decay or even absence (Van Wissen *et al*., 1991; Wojcik *et al*., 1995a). Impaired spindle function could be one of the reasons of oocyte immaturity and failure to be fertilized.

In *Xenopus* oocytes cortical and animal–vegetal gradients in proteasome distribution have been observed (Ryabova *et al*., 1994). While there are no vegetal and animal regions in the human egg and, therefore, the existence of animal–vegetal gradients is impossible, we have detected a uniform and weak cortical gradient, with proteasome concentration slightly increasing towards the cell periphery. In contrast to the salamander *Pleurodeles waltl*, proteasomes during human oogenesis are absent from the germinal vesicle (Pal *et al*., 1988).

Proteasomes in the oocyte probably not only perform proteolytic functions, but are also stored as a maternal supply of proteasomes for the developing embryo. During early *Xenopus* embryogenesis, maternal proteasomes are replaced by proteasomes expressed by the embryo itself around the gastrula stage (Fujii *et al*., 1991). A similar process also takes place at some stage of human development. It is possible that the disappearance of the maternal proteasomes is reflected by the disappearance of the peripheral cytoplasmic labelling.

While most attention is focused on the proteolytic functions of the proteasomes and, therefore, their role in degradation of different crucial substrates (e.g. cyclins), it should be kept in mind that these particles, referred to as prosomes, are also mRNA binding particles and hence their distribution and function can also imply the regulation of the storage of maternal mRNA (Grainger and Winkler, 1989; Ryabova *et al*., 1994; Schmid *et al*., 1995).

After fertilization, there appears to be a large shift in proteasome distribution, since the cortical gradient increases and they concentrate at the periphery of the zygote. It is noticeable that, in contrast to the nuclei, pronuclei do not contain proteasomes at detectable levels. It is a matter for discussion whether this shift in proteasome localization is somehow triggered by the acquisition of the proteolytic centre or proteasomes associated with sperm centrioles (unpublished results), or whether it is an independent phenomenon due to oocyte activation. This issue could be clarified by studies using animal oocytes. In the case of *Xenopus* oocytes, it has been shown that their activation induces the formation of a dorso–ventral gradient of proteasome distribution (Ryabova *et al*., 1994). Since the pronuclei are devoid of proteasomes, the female pronucleus should not allow the re-entry of ooplasm proteasomes after the end of the second meiotic division. In the case of male pronucleus, the absence of proteasomes is easier to explain, since we observed that sperm heads are devoid of proteasomes (unpublished observations). It is plausible that the proteasome concentration at the periphery of the zygote is part of a mechanism to avoid nuclear re-entry of the proteasomes, which could be mediated by a calcium/myosin-dependent mechanism as in *Xenopus* (Ryabova *et al*., 1994).

After syngamy and the first cleavage, proteasomes apparently somehow re-enter the nuclei, since we have observed them in the nuclei of a triploid 3-cell embryo and in both triploid blastocyst cells and apoptotic diploid embryos. Moreover, since the nuclear MCP21 immunolabelling is very strong, most of the embryo proteasomes are probably located there. While there is still some clear peripheral cytoplasmic labelling observed in the triploid blastomeres, the labelling disappears at the blastocyt stage. Instead, there is only a weak, diffuse cytoplasmic immunolabelling pattern. Nuclear localization of proteasomes in human embryo development contrasts with a report on *Pleurodeles* embryos, where most proteasomes were cytoplasmic, and only at later stages of embryonic development did nuclear relocalization of the proteasomes occur (Pal *et al*., 1988). In addition, in early ascidian embryos, during the interphase the proteasomes are located mainly in the nucleus, while they dissociate from the chromatin at prophase (Kawahara and Yokosawa, 1992). During early chicken embryogenesis, proteasomes are uniformly nuclear in location, while at later stages (stage 4 onwards) they are also present in the cytoplasm (Pal *et al*., 1994). At later stages of development, increased expression of proteasomal antigens is seen in the regions where extensive proliferation occurs (Hutson *et al*., 1997).

All the discarded diploid embryos that were examined have shown chromatin abnormalities, which are typical of apoptosis (Levy *et al*., 1997, 1998). This finding confirms that the selection of viable embryos for intrauterine transfer was made properly and only non-viable embryos were discarded. It is also interesting to note that the proteasomes are retained in the nuclei of the apoptotic embryos. It is possible that proteasomes do perform some role in the initiation and/or execution of apoptosis in preimplantation human embryos, as they do in other systems (Wójcik, 1999b).

While in the living triploid embryo, proteasomes and DNA co-localize in the nuclei, during apoptosis there appears to be a dissociation, and DNA domains do not always overlap with proteasome domains in the nucleus. It appears that proteasomes are not included into different condensed forms of chromatin, e.g. mitotic chromosomes, sperm heads or apoptotic bodies. This finding confirms previous reports that nuclear proteasomes are only loosely associated with the chromatin (Wójcik, 1999a).

In conclusion, this is the first report which shows the localization of proteasomes in mammalian oocytes and embryos. Moreover, we provide evidence for the existence of great changes in proteasome distribution at fertilization and further stages of development. Since we only used old oocytes or dead or abnormal embryos, it is possible that the conclusions regarding the normal developmental process inferred from our results do not properly reflect the real situation. Our studies should be complemented in the future with studies using normal animal material. Due to the requirement of unique human material, we limited our observations to a small number of oocytes and embryos. In order to obtain images from several specimens, which will allow some generalization of our findings, we limited our study to the use of only one monoclonal antibody. Further studies with the use of different antibodies recognizing not only 20S but also 26S proteasome subunits will be of interest.

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