For the Western blotting of the supernatant, the supernatant was concentrated using the Amicon Ultra 30 K centrifugal filter (EMD Millipore, Billerica, MA) according to the manufacturer's instructions. The primary antibodies used in the experiments were Phospho-Akt (Ser473), total-Akt, MMP-2, MMP-9, Phospho-STAT3, Vimentin, and MT1-MMP (Cell Signaling Technology, Beverly, MA). E-cadherin and TIMP-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin was from Sigma–Aldrich. The membranes were washed with TBST three times for 10 min each time, treated with horseradish peroxidase (HRP)-conjugated secondary antibody at RT for 1 hr, and then washed with TBST three times for 10 min each time. Specific signals were detected using the ECL or ECL Prime Kit (GE Healthcare, Tokyo) and the LAS 4000 imaging system.

Gelatin Zymography

Starved prostate cancer cells were cultured in control medium or CM with or without anti-MCP-1 antibody for 24 hr, and the medium was collected and concentrated using the Amicon Ultra 30 K according to the manufacturer's instructions. An equal volume of the sample buffer was added to the concentrated medium. After electrophoresis using 10% Zymogram (Gelatin) gel with 0.1% gelatin (Novex®; Life Technologies Japan, Tokyo) with 125 V constant for 90 min, the gel was rinsed with 100 ml of renaturing buffer for 30 min and incubated overnight in 100 ml of developing buffer at 37°C. After incubation, the gel was rinsed three times for 5 min each time with deionized waterand stained with the SimplyBlue Safestain (Invitrogen, Carlsbad, CA) for 1 hr at RT. MMP-2 and MMP-9 enzymatic activity was detected as transparent bands.

Tissue Samples and Clinical Data

Untreated human primary prostate cancer tissues were obtained during radical prostatectomies at Yokohama City University Hospital (n = 87). We have defined cancer lesions by gross findings of cut plane and confirmed pathologically. The sampling and analysis of all prostate tissues was approved by the Ethics Committee of Yokohama City University Graduate School of Medicine after obtaining informed consent from each patient. All tissues were stored at −80°C until experiment, as described [12].

Statistical Analysis

Values are given as mean ± standard deviation (SD). Significance was examined by unpaired Student's *t*-test or a one-way analysis of variance (ANOVA) following the Tukey-Kramer test. A *P*-value < 0.05 was considered significant.

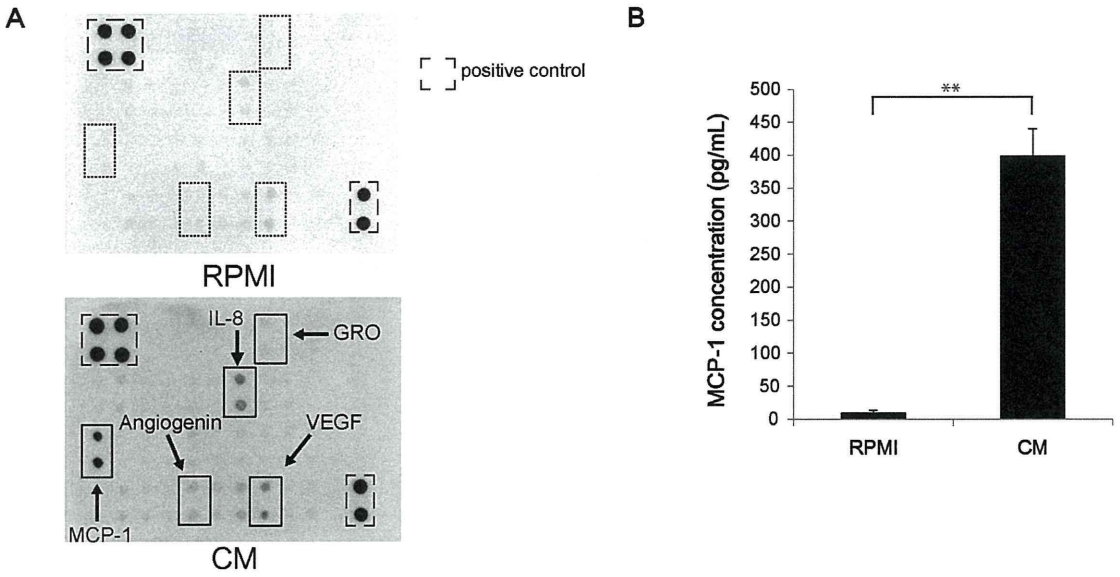

Fig. 2. Human adipocytes secrete MCP-1. (**A**) The supernatant of mature adipocytes was collected as conditioned medium (CM). The cytokines present in the CM or control medium were analyzed in a cytokine membrane array. MCP-1 showed the strongest increase in CM compared to the control medium. (**B**) The level of MCP-1 was measured by an ELISA. The data shown are the means. Error bars: SD. $^{**}P < 0.01$ according to the unpaired *t*-test.

RESULTS

The CM Induced Prostate Cancer Cell Progression

After HPAd cells were cultured in ADM for 15 days, they were differentiated to mature adipocytes (Fig. 1A). Mature adipocytes were confirmed by lipid staining using oil-red-O. We then prepared the CM and used it for the further experiments. First, we determined whether or not the CM stimulated the growth of the prostate cancer cells. After 2 days' incubation in the CM, the cell growth of each cell line was measured by in an MTT assay. As shown in Figure 1B, all cell lines treated with CM increased their growth ($P = 0.006$ in DU145, $P < 0.001$ in LNCaP, and $P = 0.042$ in PC-3). We then examined whether or not the migration and invasion activities of the DU145 and PC-3 cells were affected by CM. The CM remarkably up-regulated these activities compared to control medium. (Fig. 1C and D) These results suggest that some factors included in CM up-regulate prostate cancer progression.

The CM of Adipocytes Showed Growth Factors and Cytokines

Because the CM included various kinds of cytokines and growth factors, we next assessed the growth factors and cytokines by using a human cytokine array kit to determine which of the cytokines or growth factors are secreted. Compared to the control medium, the CM had higher secretion levels of MCP-1, interleukin-8 (IL-8), growth-related oncogene (GRO), angiogenin, and vascular endothelial growth factor (VEGF) (Fig. 2A). In particular, MCP-1 was the highest increasing protein present

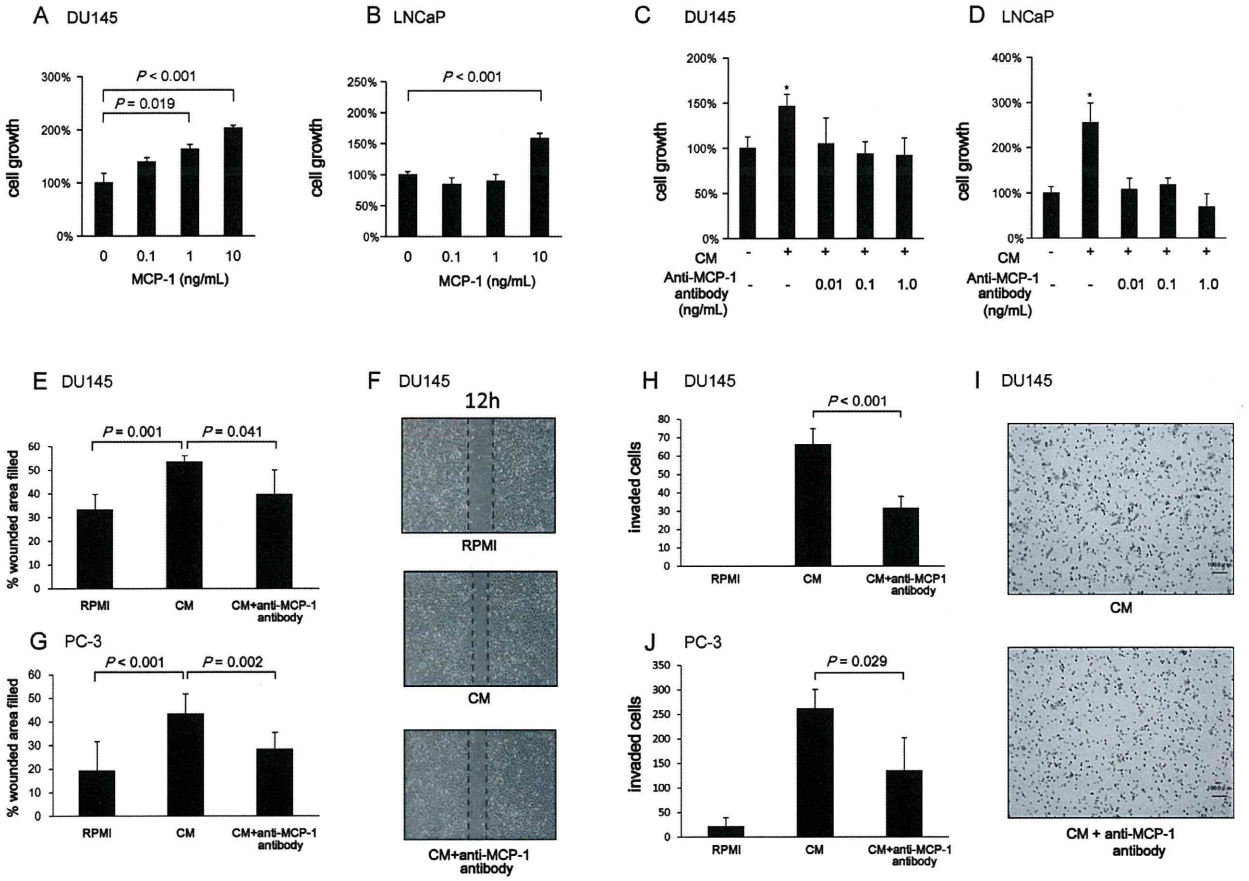

Fig. 3. MCP-1 present in the CM induced prostate cancer cell progression. (**A** and **B**) Cell growth of DU145 and LNCaP stimulated by MCP-1. Cells were seeded in 12-well plates, and four wells were used for each sample. Forty-eight hours after MCP-1 stimulation, the cell number was counted. (**C** and **D**) An MTT assay was performed in 96-well plates, and four wells were used for each sample and counted 48 hr after stimulation with CM or CM with anti-MCP-1 antibody. (**E**–**G**) Serum-starved confluent monolayers of DU145 and PC-3 cells were wounded by scratching, then incubated with CM, CM with anti-MCP-1 antibody, or control medium for 12 hr. Before stimulation, CM was pre-incubated with anti-MCP-1 antibody for 1 hr (10 μg/ml). Migration was assessed as indicated in Figure 1. (**H**–**J**) DU145 and PC-3 cells' invasion followed stimulation with CM, CM with anti-MCP-1 antibody, or control medium.

in the CM. Further, we confirmed the MCP-1 concentration in the CM by an enzyme-linked immunosorbent assay (ELISA). The MCP-1 concentration in the CM was much higher than that in the control medium (Fig. 2B, $P < 0.001$).

MCP-1 in the CM Up-Regulated the Prostate Cancer Cell Progression

Because MCP-1 showed the highest secretion in the CM, we focused on the association between MCP-1 and prostate cancer progression. The prostate cancer cell lines DU145 and LNCaP, increased their growth in a dose-dependent manner by MCP-1 stimulation (Fig. 3A and B). Further, anti-MCP-1 antibody suppressed the cell growth by CM treatment in a dose-dependent manner (Fig. 3C and D).

To verify whether MCP-1 in the CM affects cell migration, we carried out a wound-healing assay using DU145 and PC-3 cells. As shown in Figure 3E–G, the wounded monolayer in the CM recovered much more quickly than those in control medium. The percent area by CM stimulation was by1.6—2-fold larger than that of the control medium ($P = 0.001$). Anti-MCP-1 antibody significantly inhibited the effect of wound closure by the monolayer of both cells ($P = 0.041$ in DU145, $P = 0.002$ in PC-3).

To evaluate the invasion activities of DU145 and PC-3 cells, we conducted an invasion assay. Many invaded cells were found in the CM-treated group, whereas fewer invaded cells were recognized in the control medium (Fig. 3H–J). The CM with anti-MCP-1 antibody reduced the number of invaded cells by approx. 50% compared to the CM ($P < 0.001$ in DU145, $P < 0.029$ in PC-3).

Akt Phosphorylation was Induced by the CM

To assess whether CM induces Akt phosphorylation in prostate cancer cells, we conducted a Western blot analysis for Akt phosphorylation in DU145 cells. As shown in Figure 4, Akt phosphorylation by CM stimulation was induced in a time-dependent manner in DU145 cells. Akt phosphorylation was induced at 60 min by CM stimulation and blocked by anti-MCP-1 antibody.

DU145 and PC-3 Activated Pro-MMP-2 in CM and Anti-MCP-1 Antibody Inhibited MMP-2 Activation

To further clarify the invasion activities of prostate cancer cell lines mediated by MCP-1, we investigated the gelatinase activities by using a gelatinzymography procedure. The zymography of

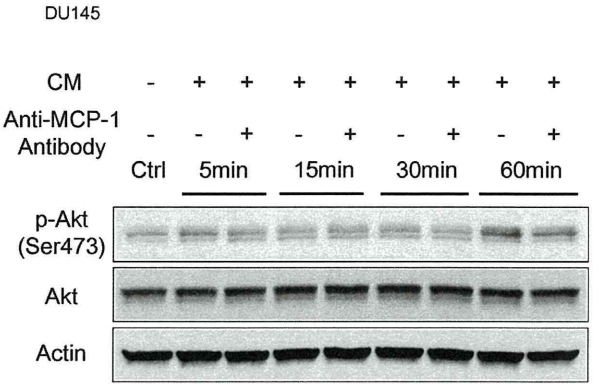

Fig. 4. The CM-induced Akt phosphorylation was decreased by the neutralization of MCP-1. Western blot assay of phosphor-Akt and total Akt in DU145 cells. Serum-starved DU145 cells were incubated with CM or CM with anti-MCP-1 antibody from 5 to 60 min. Before stimulation, CM was pre-incubated with anti-MCP-1 antibody for 1 hr (10 μg/ml). Upper blot, phospho-Akt (Ser 473); middle, total Akt; bottom, β-Actin. The data are representative of three independent experiments.

DU145 and PC-3 cells cultured in CM showed markedly activated MMP-2 (Fig. 5A, lane CM). The CM itself contained pro-MMP-2 and showed no band of activated MMP-2 (Fig. 5A, lane Ctrl). The CM with anti-MCP-1 antibody diminished activated MMP-2 band clearly. Western Blot of supernatant revealed that MMP-2 protein level was unchanged in prostate cancer cell lines (Fig. 5B). These findings suggest that CM-treated DU145 and PC-3 cells could convert the pro-MMP-2 present in CM to the active form and that MCP-1 in the CM regulates MMP-2 activation.

CCR2 mRNA Expression Correlated with Gleason Score of Prostate Cancer Tissues

C-C chemokine receptor type 2 (CCR2) is a cytomembrane receptor for MCP-1. We investigated the expression level of CCR2 mRNA in surgical specimens obtained during radical prostatectomies. The expression level of CCR2 mRNA was significantly up-regulated in proportion to the Gleason score (Fig. 6, $P < 0.01$). There was also a tendency of higher CCR2 mRNA expression in the higher pathological T stage, although it was not significant (data not shown; $\leq$ pT2 vs. $\geq$ pT3, $P = 0.068$). CCR2 mRNA expression was not found to be associated with the patient's age, body mass index (BMI), or PSA levels (data not shown).

DISCUSSION

Obesity is recognized as one of the risk factors for prostate cancer [13]; but the mechanism under-

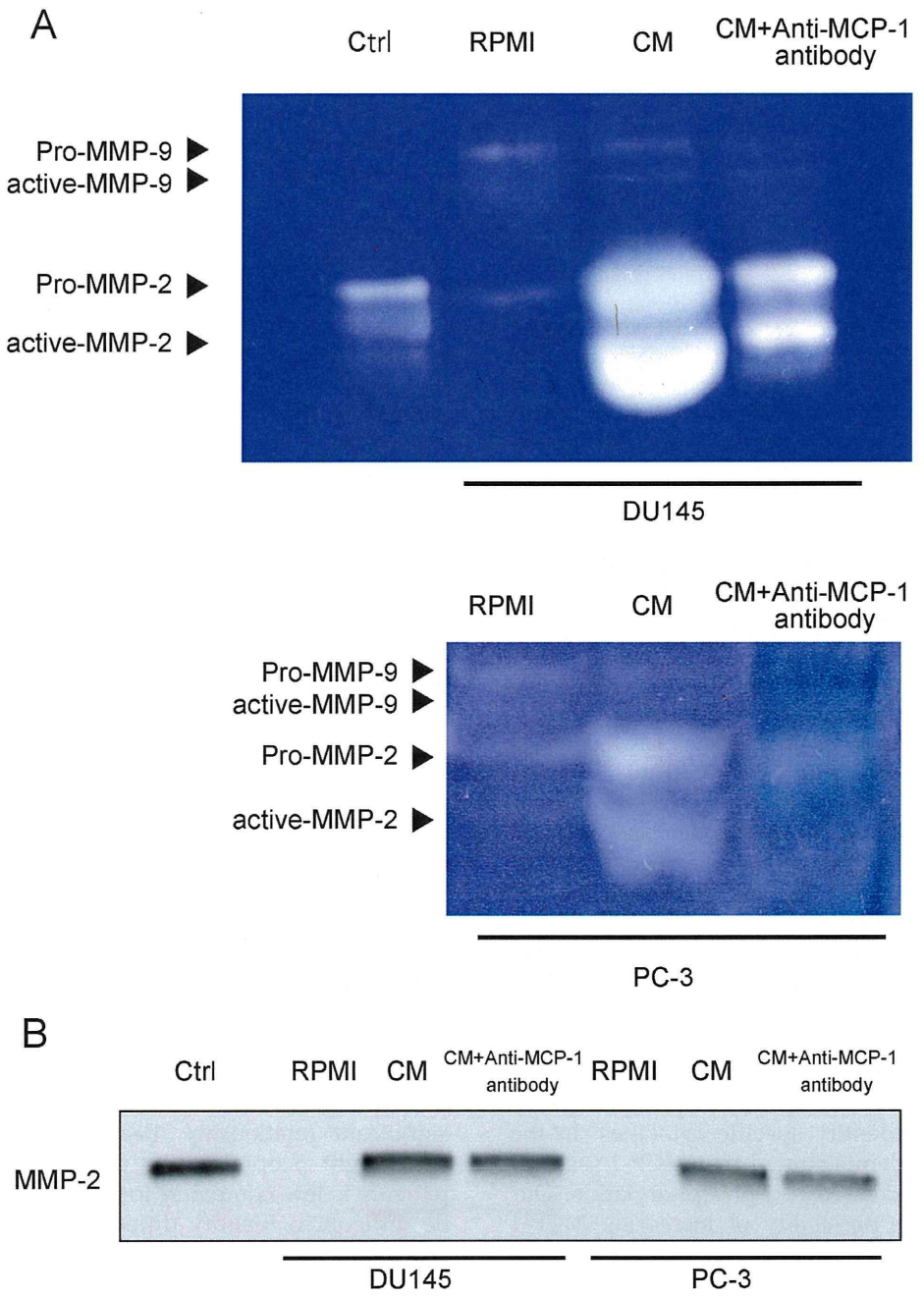

Fig. 5. MMP-2 enzymatic activity is up-regulated via CM stimulation. (**A**) Effect of CM on the enzymatic activities of MMP-2 and MMP-9 in DU145 and PC-3 cells were analyzed by gelatin zymography. Ctrl; only CM, RPMI; the supernatant of prostate cancer cells treated with RPMI, CM; the supernatant of prostate cancer cells treated with CM, CM + Anti MCP-1 antibody; the supernatant of prostate cancer cells treated with CM with anti-MCP-1 antibody. (**B**) Results of the Western blot assay of MMP-2 in the CM, control medium, and CM plus anti-MCP-1 antibody incubated in DU145 or PC-3 for 24 hr.

lying the association between obesity and prostate cancer is not known. We hypothesized that some cytokines secreted from adipocytes up-regulate the malignant potential of prostate cancer. In the present study, CM from adipocytes induced prostate cancer proliferation, migration, and invasion compared to control medium. We observed up-regulations of MCP-1, IL-8, and VEGF, among which we focused attention on the marked difference of MCP-1.

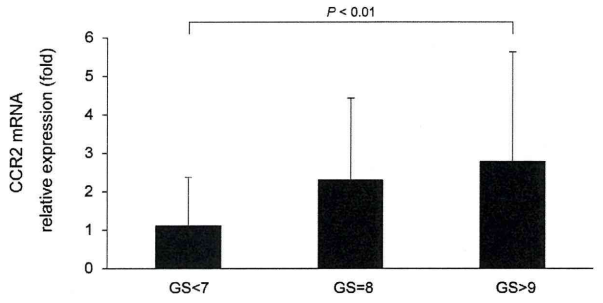

Fig. 6. CCR2 mRNA expression correlated with Gleason Score of prostate cancer tissues. RT-qPCR analysis of CCR2 expression levels in PCa surgical specimens obtained from radical prostatectomies (n = 87). The expression level of CCR2 mRNA was significantly up-regulated in proportion to the Gleason score ($P < 0.01$).

MCP-1 has been shown to be increased in the adipose tissue of obese individuals [5]. MCP-1 has various roles in cancer development and progression. The expression of MCP-1 receptor is increased in aggressive prostate cancer compared to less-aggressive and benign prostate cells [14]. Yi et al. showed that in patients with bone metastases, MCP-1 levels were significantly elevated compared to localized PCa [15]. Here we showed that the neutralization of MCP-1 in CM inhibited prostate cancer cell growth, migration, and invasion. We thus speculate that MCP-1 from adipocytes has an important role in prostate cancer progression.

MCP-1 stimulates various types of cellular signaling for cancer progression. Akt phosphorylation plays an important role in prostate cancer cell growth and survival [16–18], and MCP-1 increases Akt phosphorylation in prostate cancer cells. Huang et al. reported that a high-fat diet increases Akt phosphorylation [5], but they did not identify specific cytokines. In the present study, we demonstrated that MCP-1 secreted from adipocytes increased Akt phosphorylation. Our results showed the possibility of increasing MCP-1 involved in the progression of prostate cancer through Akt phosphorylation.

Mechanisms of prostate cancer invasion by MCP-1 have been explained with epithelial-mesenchymal transition (EMT) and expression changes of integrins. EMT is a dramatic change in cell phenotype, and it often enables an invasion by carcinoma cells. Izumi et al. reported that MCP-1 secreted from prostate cancer cells and macrophages is enhanced by androgen receptor knockdown and leads to EMT through the phosphorylation of STAT3 [19]. To evaluate the involvement of EMT, we analyzed the phosphorylation of STAT3 and the expressions of E-cadherin and vimentin by Western blotting (Supplementary Fig. S1A). The results showed no change in the phosphorylation of STAT3 or the expressions of E-cadherin and vimentin.

Lin et al. reported that prostate cancer stimulated by MCP-1 expressed high mRNA levels of integrin-αv and integrin-β3 and acquired more invasion activity [20]. However, we confirmed no escalation of integrin-αv or integrin-β3 mRNA levels in prostate cancer cells stimulated by CM (Suppl. Fig. S1B). Because various growth factors and cytokines are included present in CM, other cytokines might be involved in prostate cancer progression. We found that MCP-1 in the CM up-regulates the enzymatic activity of MMP-2 and is not involved in MMP-9 activation. In addition, the MMP-2 protein level was unchanged. This suggested the hypothesis that prostate cancer cells may alter the enzymatic activity of MMP-2 present in CM through MCP-1 stimulation.

It is well known that cancer cells control the activities of MMPs secreted by stromal cells by regulating the expression of membrane type 1-matrix metalloproteinase (MT1-MMP) or tissue inhibitor of metalloproteinase 2 (TIMP2) [21–23]. We confirmed the expression of MT1-MMP and TIMP2 by Western blotting, which showed no change by CM stimulation (Suppl. Fig. S1A). Therefore, another mechanism might be involved in the regulation of MMP-2 activation, but not through MT1-MMP or TIMP2 activation.

To clarify the relationship between MCP-1 and prostate cancer, we analyzed the serum concentration of MCP-1 from 75 prostate cancer patients by ELISA. The results indicated no differences among the BMI, serum MCP-1 concentration, and Gleason score or clinical stage (data not shown). Such an analysis should be conducted in a cohort in which the BMI, Gleason score and/or clinical stage has a mutual significant relationship. Because the proportion of overweight people among Japanese prostate cancer patients is low compared to Western patients, it may be difficult to identify differences between the BMI and pathological characteristics in Japanese prostate cancer patient cohorts. In fact, although BMI > 30 was defined as obese in the World Health Organization (WHO) BMI classification, no patient with a BMI of 30 or greater was present in our cohort. However, the CCR2 mRNA expression level was significantly up-regulated in proportion to the Gleason score in our cohort. These results indicated that high-grade prostate cancer may be more susceptible to the effect of MCP-1, leading to a metastatic or invasive stage.

In a large cohort study, Yin et al. showed that elevated BMI was associated with the risk of prostate cancer motility and recurrences [24]. Androgen depletion therapy (ADT) leads to the development of

vasomotor flushing, fatigue, osteoporosis, anemia, gynecomastia, and obesity in prostate cancer patients [25]. Keto et al. reported links between ADT, obesity and prostate cancer progression [26]. Thus, obesity caused by ADT might also induce prostate cancer metastasis, and MCP-1 secreted from adipocytes may play a part of in the malignant alteration.

In conclusion, we here obtained evidence of an association between MCP-1 secreted by adipocytes and prostate cancer progression. In addition to the inhibition of MCP-1 function, the control of obesity in men also might influence the progression of prostate cancer.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Chapter

22

PLEIOTROPIC FUNCTIONS OF MAGNETIC NANOPARTICLES FOR *EX VIVO* GENE TRANSFER AND CELL TRANSPLANTATION THERAPY

Daisuke Kami[1*], Masashi Toyoda[2], Masatoshi Watanabe[3], and Satoshi Gojo[4]

[1] Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan
[2] Department of Vascular Medicine, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan
[3] Faculty of Engineering, Yokohama National University, Kanagawa, Japan
[4] Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

*Corresponding author: dkami@koto.kpu-m.ac.jp

Contents

22.1. INTRODUCTION

Magnetic nanoparticles (MNPs) are widely used in research and medical applications and have attained new levels of versatility and functionality. In this study, we determined the feasibility of traceable *ex vivo* gene transfer using MNPs instead of a multiple-step approach involving *in vitro* gene transfer, isolation of cells, and marking for *in vivo* tracing. This is a breakthrough technology that introduces new possibilities and a novel concept for cell transplantation therapy. In this chapter, we describe the development of this concept and its future potential.

22.2. NANOTECHNOLOGY AND MAGNETIC NANOPARTICLES

Nanotechnology is the manipulation and application of nano-sized materials. These methods have already been applied in various fields. Compared to micro-sized particles, nanoparticles (approximately 100 nm) that exhibit magnetic characteristics display higher fluidity and surface area as well as improved reaction efficiency. These particles also express magnetic properties and show easily controllable behaviours; therefore, they produce a strong effect in a limited space [1].

Divalent or trivalent iron oxide is the major material used in the formulation of magnetic nanoparticles (MNPs). Iron oxide presents low cell toxicity and has been used in the clinical field as a contrast agent for magnetic resonance imaging (MRI), which will be described later. MNPs have also been prepared using cobalt, manganese, nickel, and neodymium metal oxides; however, these display stronger cytotoxicity than iron oxide MNPs, and thus need to be coated [2]. Coating substances are generally selected to reduce the cytotoxicity of magnetic particles. In many cases, dispersing agents are used as coating substances to provide additional functionality to the nanoparticles.

Researchers are currently attempting to add functionality to the surface of MNPs [2]. For example, particles coated with dispersants, which enhance dispersibility, could be unmobilised under a magnetic field. Antibodies can also be coupled to particle surfaces, allowing the effective acquisition of a target *via* interactions between the coupled nanoparticles and proteins, bacteria, or cells with epitopes for the antibody that exclusively express the specific protein (magnetic-activated cell sorting, immunoprecipitation, and magneto-microfluidics) [3]. In addition, pharmaceutical agents, such as anticancer drugs, could be coupled with MNPs and administered at a targeted site at the minimum required dosage. These complexes could then be localised under a

magnetic field, thereby reducing adverse effects to the body. MNPs could serve as an effective drug delivery system [4].

In addition, the fine vibrations caused by exposure of MNPs to an alternating magnetic field result in the generation of heat. This phenomenon, called hyperthermia, could be applied to the treatment of cancer. Recently, a system has been devised wherein MNPs are concentrated within the cancer *via* neoangiogenic blood vessels, and heat is generated using an alternating magnetic field [5].

MRI contrast agents using iron oxide have been commercially available since the late 1990s. Various diagnostic utilities of MNPs have previously been reported, and these particles are highly reliable contrast agents for normal use. MRI, using iron oxide-based contrast agents, is generally the first choice as the most effective and non-invasive technique for the diagnosis of metastatic liver cancer. Moreover, recent advances in MRI technology have given rise to new prospects for MRI use (in combination with other analytical methods).

As mentioned above, MNPs can be applied in various fields and potential for use in the medical care industry in the near future.

In this chapter, we report a novel cell transplantation treatment strategy focusing on gene transfer using MNPs.

22.3. GENE TRANSFER USING MNPs

One of the main biological applications of MNPs is their use in a gene transfer method called magnetofection [6], in which nucleic acids such as DNA (plasmids) and RNA can be transferred into cells. Such nucleic acid transfer is an important tool that is routinely used in current life science research, such as for the control of target gene expression and cell labeling. In recent years, gene transfer technology has been successfully used in the production of induced pluripotent stem cells (iPSCs) [7,8] and in Cas nuclease RNA-guided genome editing (CRISPR) [9] or transcription activator-like effector nuclease (TALEN) [10] editing, thus suggesting a further increase in its importance in the near future.

Currently, there exist three principal methods for gene transfer: (1) viral vectors, such as recombinant retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses, to deliver genomic materials into cells; (2) electroporation to disturb cell membranes by electrical stimulation and promote the passive transfer of genes of interest into cells; and (3) chemical reagents such as cationic polymers to encircle nucleic acids, fuse with cell membranes, and release them into the cytosol. Among these, the method utilising a chemical reagent is suitable for clinical applications owing to its relatively low cytotoxicity with negligible genomic incorporation. However, the gene transfer efficiency of chemical reagents is generally lower than that of

other methods. Therefore, the improvement of transfer efficiency using the reagents has been highly anticipated.

We attempted to improve the gene transfer efficiency of the transfection reagent by utilising MNPs as nucleic acid carrier agents. Divalent and trivalent iron, cobalt, manganese, nickel, and neodymium are currently available MNPs. Among these, iron oxide is used as an MRI contrast agent and is known to display a low cytotoxicity. Therefore, we used iron oxide as the nucleic acid carrier and the cationic polymer poly(ethyleneimine) (PEI) as the dispersing agent for iron oxide. PEI is a well-characterised polymer that has been used as a gene transfer reagent. Because of the commercial availability of PEI with various molecular structures, modifications, and molecular weights, the specific form of PEI can be selected according to the application. We chose deacylated PEI (PEI max, Polysciences, Inc., Warrington, PA, USA.), which displayed lower cytotoxicity and a linear form, to coat the iron oxide. This type of PEI was also more cationic than typical PEI. The MNPs composed of iron oxide and deacylated PEI display high dispersibility and cohesiveness under the magnetic field; this enables the construction of a superior magnetofection system (Figure 1, Tables 1 & 2). In addition, the low toxicity of these nanoparticles allow for the simultaneous introduction of multiple plasmids [11].

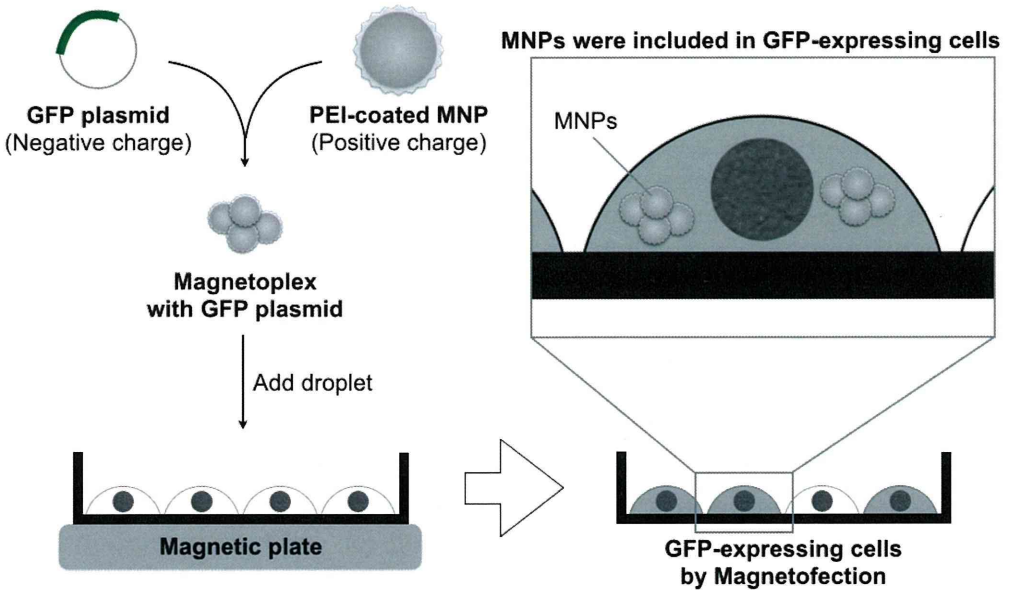

Figure 1. Diagrammatic illustration of magnetofection

Table 1. Reaction mixture of magnetofection for 6 well plate

Plasmids (1 μg/μl)	1.0 μl/well
PEI-coated MNPs (50 μg MNPs/ml)	7.5 μl/well
Deionised water or Opti-MEM*	41.5 μl/well
Total **	50.0 μl/well

* Life Technologies, Inc.

** Mixtures were reacted for 15 min at room temperature

Table 2. Transfection efficiency of our magnetofection method

Cell line	Species	Description	Transfection efficiency	References
P19CL6	Mouse	Embryonic carcinoma	81 %	[12]
MEF	Mouse	Embryonic fibroblast	9 %	[13]
HeLa	Human	Cervical cancer cell	40 %	[14]
TIG-1	Human	Fetal lung fibroblast	--	[11]

The gene transfer efficiency of this magnetofection system depends on the size of the magnetoplex, which is comprised of MNPs and plasmids. Cationic polymer PEI-coated MNPs (positive charge) and nucleic acids (negative charge) are electrodynamically coupled to form the magnetoplex complex [14]. The size of the magnetoplex complex varies according to the amount of MNPs used. A larger quantity of MNPs results in a larger number of nucleic acids that are coupled and a larger magnetoplex. The gene transfer of MNPs is dependent on endocytosis, the mechanism by which the magnetoplex enters the cell. The larger the size of a magnetoplex, the poorer the efficiency of gene transfer [14]. This trade-off is quite important to establish an optimal condition for magnetofection, which depends upon the host cells.

22.4. STRATEGIES FOR THE DEVELOPMENT OF A NEW METHOD FOR CELL TRANSPLANTATION THERAPY USING MNPs

Based on the properties described above, we reported a concept that could assist in the development of a new method for cell transplantation therapy using magnetofection in 2014 [13]. The transfection of genes into cells using magnetofection involves the capture of MNPs within the cells (Figure 1). A chronological quantitative measurement of the residual amounts of nanoparticles within the cells using an inductively coupled plasma mass spectrometer demonstrated no significant changes in the number of MNPs per cell over a two-week period. In addition, as the cells detached by trypsinisation

reacted to the magnetic force, it was possible to control their dynamics within the solution. Based on these findings, we hypothesised and attempted to demonstrate the separation and purification of magnetofection-treated gene transfected cells using magnetic force, and the tracing of these cells *in vivo* using MRI after grafting into mice. This strategy can be effectively applied to (1) highly efficient gene transfer, (2) the separation and purification of cells by magnetic force (*in vitro* cell separation), and (3) imaging of the transplanted cells *in vivo* using MRI (Figure 2). The pleiotropic roles of MNPs are easily applied to a single system for cell transplantation therapy.

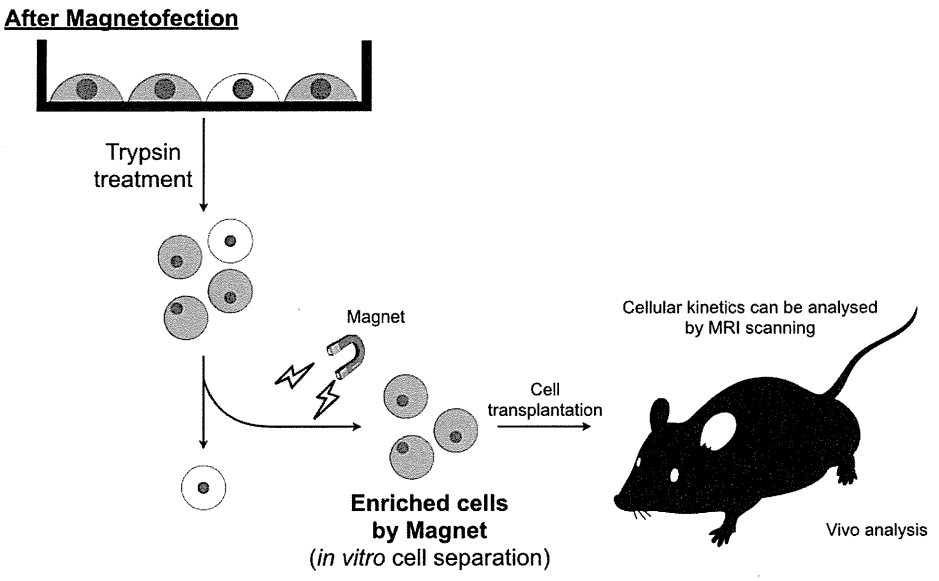

Figure 2. Diagrammatic illustration of the new strategy for cell transplantation therapy using MNPs

Because magnetofection introduces a large amount of nucleic acids into cells, the cells are subjected to a high degree of cytotoxicity. However, it has been discovered that MNPs themselves exhibit almost no cytotoxicity, and that the level of cytotoxicity increases with the quantity of nucleic acids in a dose-dependent manner. Future research should aim to optimize the cytotoxicity and gene transfer efficiency of these nanoparticles. According to our current preliminary data, a high gene transfer efficiency and low cytotoxicity can be achieved using approximately half the conventional nucleic acid quantity. Because a high transfer efficiency can be achieved using a small quantity of

nucleic acids, there is a low chance of nucleic acid insertion into the genomic sequence of host cells, which is an advantage for cell transplantation. These results will be summarised in the future.

22.5. FUTURE DEVELOPMENT

Cell transplantation therapy is currently being carried out worldwide, with many research groups utilising autologous somatic stem cells [15]. A major reason for the transplantation of cells into a patient following *in vitro* cell culture and proliferation is to bypass ethical issues and immune rejection of cell transplantation [16]. Moreover, the secretion of paracrine cytokines has been suggested to be the major mechanism influencing the efficacy of the transplanted cells. However, transplanted cells display individual secretory properties with respect to cytokines, exosomes, microRNA, and so on [17,18]. There also exist cases with few targeted cytokines or low secretion of exosomes and microRNA, since it is quite difficult to verify the quality of donor cells. This would cause variation in the overall therapeutic effect. In addition, owing to the difficulties associated with cell tracing after transplantation, it would not be possible to determine the amount of time that cells would remain in a required site.

The novel concept of cell transplantation therapy reported in this chapter offers a solution to these problems. By introducing targeted nucleic acids into cells using MNPs, cells with stable characteristics can be produced. Furthermore, transfected cells can be purified using magnetic force. Since the dynamics of these purified cells can be observed non-invasively using MRI even after transplantation into the target organs, it would be possible to assess the resulting cell behaviour. This allows the standardisation and better management of cell transplantation therapy, a process for which quality control was previously thought to be difficult. This strategy is a good example of theranostics using nanotechnology.

It is likely that the modification of nanomaterials using various techniques can provide added value to cell transplantation therapy in the future. These techniques may represent a breakthrough, reviving the currently stagnant cell transplantation therapy field.

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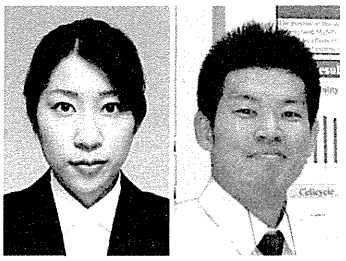
前立腺がん治療へのナノ粒子の応用

Application of nanoparticles in prostate cancer treatment



岩﨑有由美(写真左)　岡本大樹(写真右)　遠藤宣広　渡邉昌俊
Ayumi IWASAKI, Daiki OKAMOTO, Yoshihiro ENDO and Masatoshi WATANABE
横浜国立大学大学院工学府機構発現工学専攻物質とエネルギーの創生工学コース，
同工学研究院機能の創生部門医工学

◎ナノマテリアルのひとつであるナノ粒子のなかで，超常磁性体酸化鉄ナノ粒子(SPIONs)は医療へのさらなる応用が期待されているが，その曝露による潜在的なリスクを理解する必要性もある．本稿では，前立腺がんの治療，とくに去勢抵抗性およびその後のドセタキセル抵抗性前立腺がんの治療におけるナノ粒子医学の応用，およびSPIONsであるFe_3O_4磁性体ナノ粒子を併用した抗がん剤ドセタキセルの前立腺がん細胞への効果に関する基礎的研究を紹介する．二酸化チタンやフラーレンなどの他のナノマテリアルと異なり，Fe_3O_4磁性体ナノ粒子が前立腺がん細胞株においてROS産生を増加させることを見出し，酸化的DNA損傷の指標のレベルである8-OHdG産生の上昇も確認した．Fe_3O_4磁性体ナノ粒子とドセタキセルの組合せにより，ドセタキセル単独よりも前立腺がん細胞株の細胞生存率を低下させた．ドセタキセルの副作用を減らしつつ，温熱-化学療法などを組み合わせて治療効果を高めるという可能性を示したと考えられる．

Key word：超常磁性体酸化鉄ナノ粒子(SPIONs)，前立腺がん，抗がん剤，セラノスティクス

ナノメディシン(Nanomedicine)

ナノテクノロジーは，ナノスケール(10億分の1 m)の領域において原子・分子レベルで物質を制御する技術であり，素材，バイオ，医療などの産業の基盤にかかわる21世紀のもっとも重要な技術のひとつである．サイズをナノレベルにすることで，物理的・化学的そして生物学的にユニークな特徴を示すことになる(http://www.nano.gov/)．このナノテクノロジーの医学・医療分野への応用がナノメディシンであり，診断・治療に大きな発展をもたらすと考えられている．そのなかで，がんの診断・治療にかかわる分野がナノオンコロジーである[1]．診断分野ではバイオマーカー，細胞動態，分子シグナル伝達を生体で画像装置を用いてとらえることができる．治療分野では，ナノテクノロジーを利用した薬物送達システム(drug delivery system：DDS)により各種治療の効果を高めたり，使用されている抗がん剤の副作用を軽減させたり，診断と治療の2つの目的をもつセラノスティクス(theranostics)の展開に大きな影響を及ぼすと考えられる[2]．

超常磁性体酸化鉄ナノ粒子(SPIONs)の医療への応用

今回の特集のナノ粒子(nanoparticles)は，ナノテクノロジーを支える重要な柱であるナノマテリアルのひとつである．ナノマテリアルはすくなくとも一次元の大きさが100 nmよりも小さく，一次元がナノスケールの薄膜・塗膜，二次元がナノスケールのナノワイヤ・ナノチューブ，三次元がナノスケールの粒子より構成される．また，その素材により炭素系，金属系，セラミックス系，有機高分子系などに分けられる．ナノマテリアルはバルク材料にない，微細化による表面活性と反応

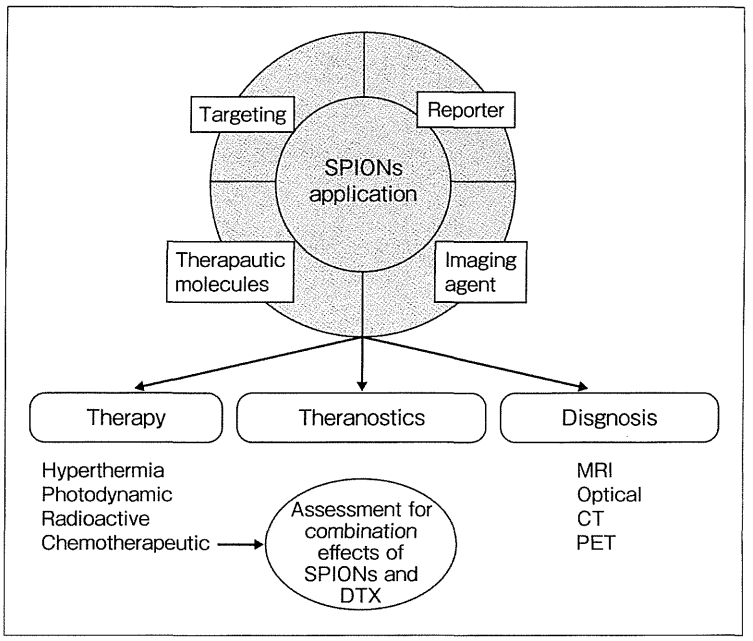


図 1　超常磁性体ナノ粒子(SPIONs)の医療への応用

性の向上と電磁気的・光学的・機械的などの物理化学的特性をもつ.

がんの領域で，ナノ粒子は診断，薬物送達，遺伝子導入，温熱療法，光線力学的療法，化学療法などへの応用，とくにセラノスティクスのために多機能ナノ粒子が研究されている[2]．ナノ粒子の素材には酸化鉄，金，銀，シリカ，ガドリニウムなどが使用されている．そのなかで，超常磁性体酸化鉄ナノ粒子(superparamagnetic iron oxide nanoparticles：SPIONs)が注目を浴びている．医療分野へ応用される理由として，原材料が安価，大量合成可能，サイズコントロールが可能，粒径分布の狭い粒子が合成可能，化学的に安定，磁化が比較的大きいことなどや，MRI造影剤(フェリデックス®やリゾビスト®)の主成分として人体内に導入する薬事承認を受けていることがあげられる[3]．さらに治療・セラノスティクスとして利用するうえで，比表面積が大きく，生体分子を多量に結合させることができることや，数十nm以下のサイズで超常磁性体を示すことなどの特性をもつためと考えられる.

超常磁性体ナノ粒子には，γ-Fe_2O_3 (maghemite)，Fe_3O_4(magnetite)，α-Fe_2O_3 (hemite)があり，コアの直径が10～100 nmである．銅，コバルトなどの他の遷移金属と混合したフェライトも超常磁性を示す．これらSPIONsが診断・治療へ応用するためには，①Enhanced permeation and retention(EPR)効果を利用したパッシブターゲティング，②表面修飾したSPIONsを利用したアクティブターゲティング，③外部磁場を利用したアクティブターゲティング，という標的指向性が求められる[4,5]．その結果として診断，治療(DDS，温熱療法，遺伝子治療など)に応用される(図1)．現在までに，メトトレキサート(methotrexate：MTX)やトラスツズマブ(trastuzumab)，ドキソルビシン(doxorubicin：DOX)を結合させたSPIONsや，DOXを結合させ，64CuでラベルしたSPIONsが開発されている[2].

期待されるSPIONsを利用したナノメディシン(ナノ粒子医学)であるが，標的指向性が低いため，さらなるリガンドのサイズや質の検討が必要である．また，基本成分であるSPIONsは造影剤として承認されているが，さまざまな細胞の種類に対していろいろな特性を有するSPIONsの細胞毒性，遺伝毒性やバースト現象などの解決すべき問題がいぜんと存在する[4,5].

前立腺がんを標的に

前立腺がんはアメリカの男性に発生するもっと

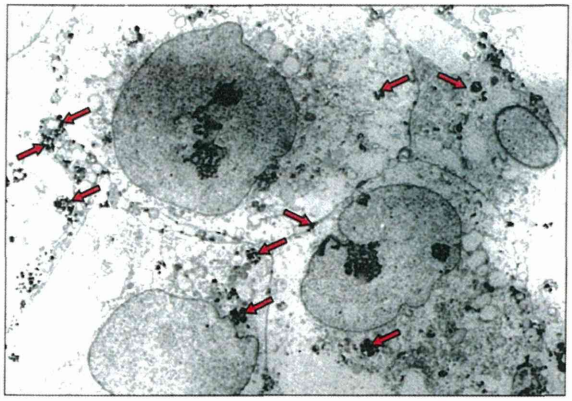


図 2　Fe_3O_4磁性体ナノ粒子の前立腺がん細胞内への取込み電顕像
赤矢印の先.

も一般的ながんであり，がん死の第 2 位を占めている．一方，日本では前立腺がんの罹患率や死亡率は低かったが，食生活の欧米化，高齢者の増加，PSA 検診の普及，診断能の向上などにより増加傾向を示し，2020 年にはその罹患者数は日本人男性のがんの第 2 位になると予想されている[6]．前立腺がんは，発生・進展にアンドロゲンとその受容体であるアンドロゲン受容体(androgen receptor：AR)がかかわるホルモン依存性のがんであることや，その発生頻度に地理学的な差異を認めるなどの特徴をもつがんである[7]．

前立腺がんの治療法には，手術療法，放射線療法，内分泌療法，待機療法があり，TNM 分類，発見時の PSA 値，生検組織における Gleason score などの要素から，最適と考えられる治療法が選択される．

前立腺がんの薬物療法には，内分泌療法と化学療法がある．病期Ⅲ期では内分泌療法を単独あるいは放射線療法と組み合わせて行い，病期Ⅳ期では主として内分泌療法を行う．また，治療効果を高める目的で，手術や放射線療法の前に，あるいは後に内分泌療法を併用することもある．有転移患者は多くの場合で内分泌療法が無効，すなわち去勢抵抗性となる．このような内分泌療法が無効となった去勢抵抗性前立腺がんに対しては，化学療法もひとつの選択肢である．しかし，化学療法は長期間使用すると副作用を伴ううえに，抗がん剤抵抗性を獲得する．このように去勢抵抗性を獲得すると長期生存は難しい．

去勢抵抗性の獲得機構は，①抗アポトーシス遺伝子の発現亢進などのアンドロゲン非依存性経路の関与，②AR 発現上昇，AR 遺伝子変異，AR スプライシンバリアント，③AR 共役因子の発現変化，副腎・前立腺内でのアンドロゲン合成経路の活性化などのアンドロゲン依存性経路の関与により生じるとされ，多くの去勢抵抗性前立腺がんにおいて，低アンドロゲン環境下の AR の異常活性化が主因と考えられている[8,9]．

このような背景の下に 2014 年，去勢抵抗性前立腺がんに対して小分子 AR アンタゴニストであるエンザルタミド(enzalutamide)，コレストロールからアンドロゲン合成を行う合成酵素である cytochrome P450-17(CYP17)の阻害剤であるアビラテロン(abiraterone)，および多剤耐性にかかわる P 糖蛋白による細胞外に排泄されにくい次世代のタキサンであるカバジタキセル(cabazitaxel)という新薬が続々と発売された[9]．

サイドメモ

前立腺がんと酸化ストレス

活性酸素種(reactive oxygen species：ROS)は好気的生物のエネルギー代謝の過程で生じる反応性の高い酸素種であり，これらを解毒・除去することが必須である．一方，細胞シグナル伝達におけるセカンドメッセンジャーとして作用し，細胞の成長および生存など多様な生物学的プロセスに関与している．酸化ストレスは ROS 産生と抗酸化防御機構との細胞内のアンバランスである．増大した ROS はがんの発生および進展に関係するといわれる．とくに，前立腺がんの発症年齢からも慢性的な ROS 産生・曝露が前立腺がんの発生・進展にかかわると報告されている．酸化ストレスから前立腺がん治療を考えたときに，蓄積した ROS に対する抗酸化剤を利用した治療より，むしろ ROS 産生を減少させる治療が望ましいと提言されている．一方，急性の過剰な ROS は細胞死を誘発することより，化学療法，放射線療法，光線力学療法が望ましいと考えられている．このように ROS の二面性はがん治療の戦略な開発につながっている．

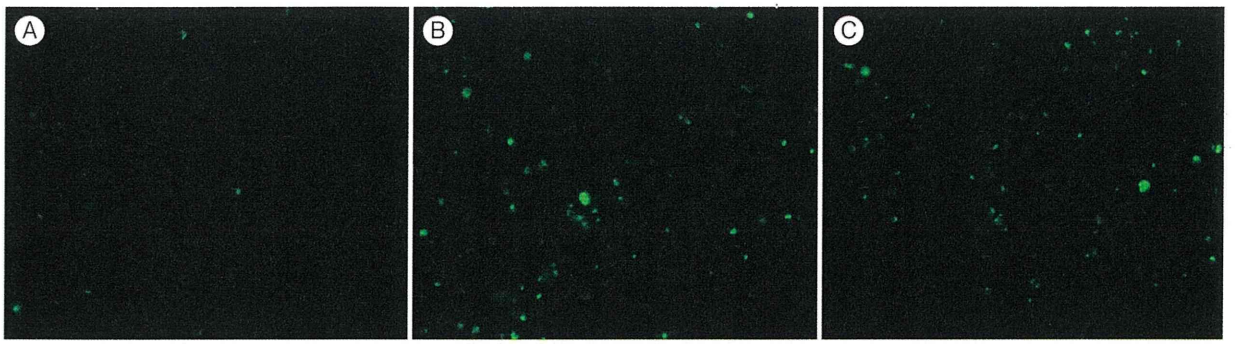


図 3　前立腺がん細胞におけるFe_3O_4磁性体ナノ粒子曝露による活性酸素種の発生
A：コントロール，B：Fe_3O_4磁性体ナノ粒子(100 μg/mL)曝露時，C：H_2O_2(100 μM)曝露時．

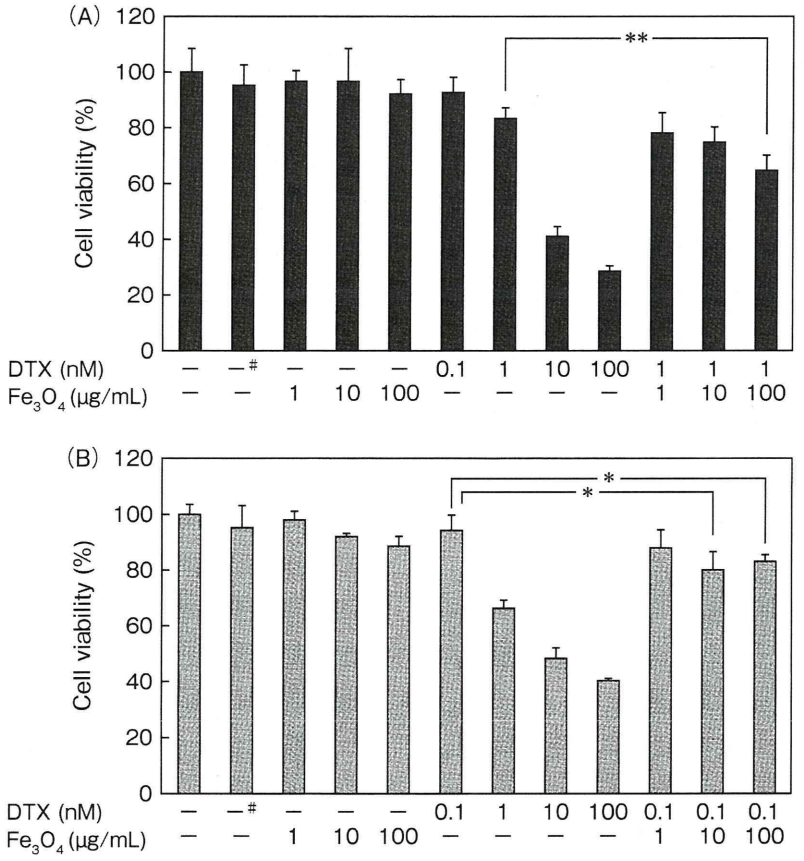


図 4　Fe_3O_4磁性体ナノ粒子とDTX併用による前立腺がん細胞株の生存率[12]
A：DU145，B：LNCaP．
*：$p<0.05$，**：$p<0.01$，#：addition of DMSO．

ドセタキセル抵抗性前立腺がんに対するナノ粒子メディシン

去勢抵抗性前立腺がんの標準治療はドセタキセル(docetaxel：DTX)を用いた化学療法である．DTXはプレドニゾロン(prednisolone：PDN)との組合せで，去勢抵抗性前立腺がんの生命予後を改善させる治療として認められている．しかし，用量依存性の副作用やドセタキセルに対する治療抵抗性の獲得などの問題がある．ナノ粒子による薬物送達システムは前立腺がん組織へのドセタキセルの透過性を高め，また維持することにより治療効果を改善し，標的外組織への毒性を減少させるのに役立つと考えられている．ナノプラットフォーム(リポゾーム，ポリマーナノ粒子，コンポ