EpitopeMap visit report

Nanoparticles' surfaces determine their impact on the cell cycle

Introduction

Engineered nanoparticles constitute promising vehicles for targeted therapies because they can cross biological barriers, enter cells and interact with them in ways that drugs alone are unable to. In principle, nanoparticles can be tailored for smart targeting of drugs, where the systems would deliver exclusively and efficiently to the chosen cell type. Therefore, the interactions of nanoparticles with biological systems are the subject of growing interest.

In recent years the challenging reality that physiological conditions represent has been revealed, suggesting that simple targeting strategies may not be effective. It is now clear that when nanoparticles come into contact with biological fluids it is not their pristine surface that interacts at the biological interface, but instead the layer of macromolecules adhered to it determines their biological fate.[1]Studies on the protein corona have shown that its composition is highly dependent on nanoparticle's physicochemical properties. For instance, it has been found that nanoparticle's composition, size and surface modification determine the presence and abundance of the proteins that will adhere to the nanoparticle[2]. Therefore, it is only natural to expect biological responses to vary according to the surface -and thus protein corona- of the nanoparticle that is exposed to cells.

The project

We have found that nanoparticles of the same size and composition can elicit very different responses on cells depending on the surface modification and/or surface charge. Commercially available carboxylate-modified polystyrene nanoparticles are routinely used as model nanoparticles to address complex biological questions since they are relatively easy to disperse in cell growth medium. Additionally, they offer the great advantage of being fluorescently labelled, which enables the use of various fluorescence-based techniques for the study of their interactions with, and uptake by, cells. Upon exposure to cells, 40nm carboxylate-modified nanoparticles readily enter the cells and after some incubation time, they can be found inside lysosomal compartments. Although the exact mechanism for their uptake remains unravelled, we have so far found no evidence for their export outside the cells once internalised[3]. Surprisingly, nanoparticle accumulation inside the cells seems to have little or no deleterious effect on the cells over the timescale of several days. In this scenario, we have observed that nanoparticle load increases with exposure time and gets diluted with cell division. We have modelled the kinetics of nanoparticle accumulation and have shown that cell division alone is sufficient to account for the kinetics observed experimentally: an initially linear increase of the

intracellular load, followed by a characteristic plateau that coincides with population doubling time of the cell line used.

Interestingly, while we have established that the carboxylate-modified nanoparticles have no acute toxic effects on cells, a very different story can be found when the amino-modified version of the same nanoparticle is used. Several reports have previously shown the potential of these cationic nanoparticles to induce cell death[4, 5]. However, when the dose administered is lowered, cell death is not predominant and instead cells can be grown in the presence of the nanoparticles for several days. Under these conditions, we have observed that nanoparticle uptake also takes place upon exposure but interestingly, its kinetics can already be deemed as different: intracellular concentration increases linearly over a longer time compared to carboxylate-modified nanoparticles and also levels-off later than the population doubling time. Coincidentally, we have found that under these conditions cell proliferation is halted, as shown by monitoring of cell numbers and also dilution of fluorescently-labelled DNA among daughter cells. We have also observed that the populations' DNA content distribution (obtained by total DNA staining) evolves differently than that of untreated cells and more importantly, cells incubated with the same dose of carboxylate-modified nanoparticles. An increase in the G2/M phase and a decrease in the S phase subpopulations were detected, suggesting that the treatment with amino-modified -but not carboxylate-modified- nanoparticles disrupted the normal progression of the cell cycle. We further confirmed this by a double-staining flow cytometry-based technique that allowed us to track in real time the progression of a given population of cells along the cell cycle as it takes up the nanoparticles. Only the treatment with the cationic nanoparticles was found to arrest the cells after 24 hours of exposure.

The cell cycle comprises the series of events during a cell's life that will lead to its duplication, and disruption of the regulation of this cycle is at the origin of carcinogenesis and cell death. Therefore, organisms possesscomplex regulatory systems to ensure the successful completion of each phase of the cycle and prevent aberrations[6]. Professor Marcos Malumbres has long worked in the area of cell cycle regulation and his group has published extensively on the topic[7, 8]. They have contributed significantly to the elucidation of the key players in the molecular pathway of cell cycle regulation, as well as the interaction between them under normal and disturbed conditions[9, 10].

Objectives

The general aim of this project was to understand how nanoparticles that differ in one of their physicochemical properties (for instance their surface modification), and thus their absorbed protein corona, can have markedly different effects on cells, in particular on their cell cycle.

One of the mainobjectiveswas to study the molecular mechanisms behind the nanoparticle-induced cell cycle arrest. In particular we were interested ininvestigating possible alterations of the expression and/or function of the proteins that are key to the regulationof the cell cycle, such as Cyclins,Cyclin-dependent kinases and inhibitors of Cyclin-dependent kinases, among others.

Another objective of the project was to gain further understanding on the nature of the cell cycle arrest. It is known that the progression through the cell cycle is tightly regulated by different molecular mechanisms,such as the two checkpoints thathave long been studied by many. In many cases where deleterious stimuli interfere with the progression of the cell cycle, it is halted at one or both checkpoints so that further errors in the cell cycle do not occur. Therefore, we were interested in studying which of the cell cycle checkpoints were activated upon exposure to the amino-modified nanoparticles.

The origin of the cellular signal that triggers the cell cycle arrest response was also among the interests of the project. We have learnt from our previous studies that exposure to amino-modified nanoparticles induces damage on the lysosomal membranes, compromising their integrity and potentially promoting the leakage of lysosomalcontent into the cytosol. It is known that the presence of lysosomal proteases in the cytosolic compartment can trigger cellular responses such as the apoptotic cascade that leads to cell death. Whether the effect on lysosomes induced by the nanoparticles is connected to the cell cycle response observed in our system was an issue of interest in the project.

Work carried out during the visit

During the first stage of the visit to Professor Malumbres' laboratory at the CNIO in Madrid (Centro Nacional de InvestigacionesOncologicas), the focus of the work was set on elucidating in which of the cell cycle phases the nanoparticle-induced cell cyclearrest took place. Moreover, since the G1/S and the G2/M checkpoints are regulated by different proteins, this was a sensible starting pointthat would allow us to focus on the relevant set of proteins during the second stage of the work. For this purpose, we carried out synchronisation experiments in which the cells were stopped at a given phase and subsequently released into the next phase in presence or absence of the amino-modified nanoparticles. In all of these experiments the carboxylate-modified nanoparticles were used in parallel as a negative control. The G1/S and the G2/M transition were both investigated with this approach, using classical synchronisation techniques and appropriate cell cycle markers to visualise and quantify by flow cytometry the percentage of cells that got halted at or progressed through the checkpoints. In this way it was possible to observe that the transition from G1 phase into S phase was markedly affected by the treatment with the amino-modified nanoparticles alone, while the G2/M transition seemed rather unaffected.

Having identified the cell cycle checkpoint that the treatment with the amino-modified nanoparticles affected, we focused on investigating possible alterations on the level of expression and/or function of the proteins that are relevant to the G1/S transition. The phosphorylation of the Retinoblastoma protein (Rb) is a key feature of the entry into S phase, for it allows the expression of a set of genes that are regulated by the E2F transcription factor and are needed for DNA synthesis during S phase. The transition between G1 and S phases is also driven by Cyclins D and E, which activate the Cyclin-dependent kinases (Cdk) 4 and 6. The level of expression of these and other proteins responsible for the cell cycle regulation were examined by two different strategies. Western blotting was used to analyse the level of the key cell cycleproteins (which reflects protein expression but also stability)after differentexposure time to the nanoparticles, while RNA sequencing was used to study the effect of the treatment at the transcriptional level in a high-throughput manner. This technique will allow us to gain a broader view of the processes that the cells are undergoing upon exposure to the nanoparticles. We hope that the RNA sequencing results will provide some insight on the cellular signal triggering the cell cycle arrest, for instance on the possibility that the arrest and the observed lysosomal damage would be connected in some way.

Main results obtained

Synchronisation of A549 (human lung carcinoma epithelial) cells with nocodazole followed by a mitotic shake-off resulted in fractions of cells enriched in mitotic cells, due to the effect of the drug on microtubule polymerisation. By releasing the synchronised cells from mitosis into G1 phase in the presence of amino- or carboxylate-modified nanoparticles we were able to assess the progression of cells through the G1/S transition. The entry into S phase was monitored by the incorporation of the nucleoside analogue EdU (5-ethynyl-2'-deoxyuridine) which was added in the cell culture medium together with the nanoparticles at the moment of release from synchronisation. EdU is an appropriate marker for S phase because it is incorporated by cells during active DNA synthesis, a hallmark of the S phase. As expected, control cells as well as cells treated with carboxylate-modified nanoparticles behaved similarly, entering the S phase at 9 hours from release (indicated by an increase in the percentage of EdU-positive cells). In contrast, treatment with the amino-modified nanoparticles dramatically delayed the entry into S phase, only showing a very small increase in EdU-positive cells 15 hours after the release (Figure 1). These results provide clear evidence that exposure to amino-modified -and not carboxylate-modified- nanoparticles affects the G1/S checkpoint of the cell cycle, inhibiting the entry into the S phase.

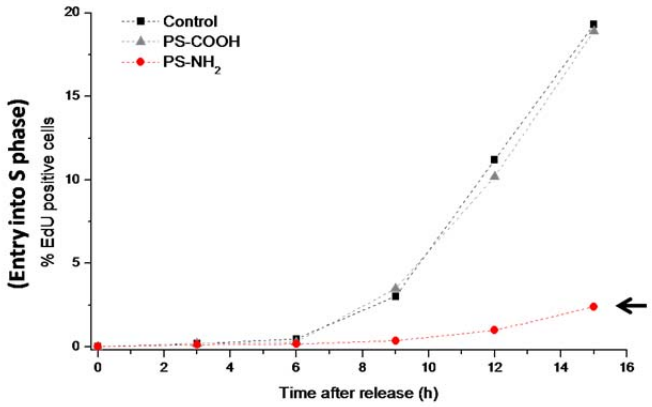

Figure 1 – Amino-modified polystyrene nanoparticles affect entry into S phase. Cells were synchronised with nocodazole in M phase and released in presence of the different nanoparticles treatments and EdU which is used as a marker for S phase.

Using a similar approach, the G2/M boundary was examined. Cells were synchronised using a classical protocol of double thymidine block which inhibits DNA synthesis and consequently stops cells in the S phase. At the moment of release from the S phase into G2 and M phase, amino- or carboylate-modified nanoparticles were added to the medium. The entrance into mitosis was monitored by immunostaining with an anti-MPM2 antibody and measured by flow cytometry. Cells incubated with amino-modified nanoparticles exhibited a response comparable to that of control cells as well as cells treated with carboxylated nanoparticles, only differing slightly in the kinetics of entry to and exit from M phase (observed as MPM2-positive cells, Figure 2). Although cells treated with the amino-modified nanoparticles entered M phase slightly later and in a less synchronised manner than the controls, the difference is not large enough and that the aminated nanoparticles do not affect the G2/M checkpoint.

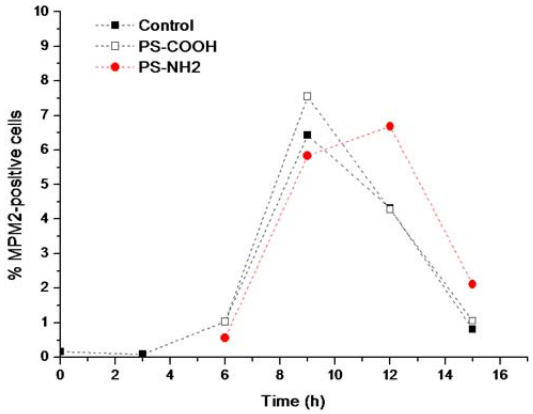

Figure 2 – Amino-modified polystyrene nanoparticles do not affect entry into M phase. Cells were synchronised with a double thymidine block in S phase and released in presence of the different nanoparticles treatments. Immunostaining with MPM2 antibody was used as a marker for mitotic cells.

Results obtained previous to this EpitopeMap visit showed cells distributed among all DNA contents in the DNA histograms of non-synchronised populations treated with the amino-modified nanoparticles. We had hypothesised that such scenario could be compatible with both cell cycle checkpoints being affected by the nanoparticle treatment. According to the results obtained with the synchronisation experiments described here that show that only the G1/S checkpoint is initially affected by the nanoparticles, we suggest that the G2/M transition would be affected later on as a secondary effect, ultimately leading to a distribution of cells with all DNA contents in the DNA histogram. Because the cells used in the experiments are cancer cells and these often have weakcell cycle checkpoints, it is possible that despite the presence of the nanoparticles that blocks the G1/S boundary some cells would have managed anyway to enter the S phase and progress into the G2 phase. However, such cells would carry replication errors that could result in problems arising during the G2/M checkpoint, leading to an apparent G2/M block as well.

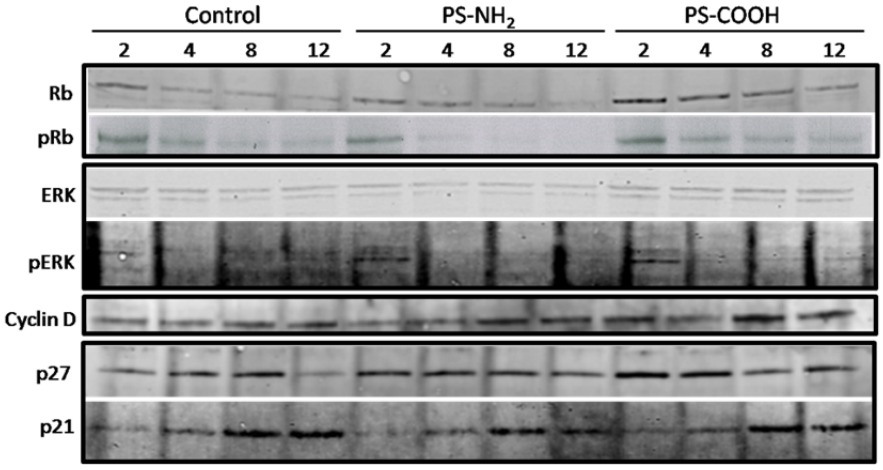

Figure 3 – Western blots of cell cycle proteins after treatment with amino-modified nanoparticles.

Knowing that the nanoparticles of interest strongly affect the G1/S but not the G2/M checkpoint, we focused on studying the proteins that are relevant for the regulation of the G1 and S phases of the cell cycle. The analysis of protein levels were performed using extracts of cells that had been synchronised with nocodazole prior to the addition of nanoparticles at the moment of release, as described before, to obtain a cleaner and stronger signal than with a heterogeneous, non-synchronised population of cells. The most striking result obtained in these analyses was the decrease in the level of phosphorylation -but not the total protein- of the Retinoblastoma protein (Figure 3). The phosphorylation of Rb is necessary for the entry into S phase and thus its decrease is consistent with the inhibition of DNA synthesis induced by the nanoparticle treatment. Interestingly, no major changes were observed in the levels of the following proteins: Cyclin D, p21, p27, ERK/MAPK, phospho-ERK/MAPK, p38, phospho-p38.

The unchanged levels of Cyclin D suggest that the cell cycle arrest could possibly be due to an effect on the activity of the corresponding Cdks. Activity assays of Cdk 4 and 6 in the presence of the amino-modified nanoparticles are among the following steps that we would like to carry out in collaboration with the host institution. We believe that the results from the RNA sequencing of RNA extracts of cells exposed to the nanoparticles after nocodazole synchronisation will shed some light on the underlying cellular processes to the observed cell cycle arrest. RNA sequencing experiments were still on-going at the moment of writing this report.

Among the future studies that we would like to carry out in collaboration with Professor Malumbres' group is the study of the nanoparticle-induced cell cycle arrest in primary cells where we can specifically ablate key cell cycle regulators such as p53 and Rb, to investigate the dependence of the effects with these proteins.

Projected publications

It is envisaged that a publication will be written in collaboration with the host laboratory in order to compile the results that were generated during this EpitopeMap visit. The focus of such article will be the molecular mechanisms underlying the nanoparticle-induced cell cycle arrest, with particular emphasis on the effect on the G1/S checkpoint and the inhibition of DNA synthesis. We hope to continue to work on this project in collaboration with Professor Malumbres' laboratory so that we can gain further insight on the cellular signal that is responsible to the downstream effects that we observe.

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