Does Adenosine A_{2A} Receptor stimulation modify axon guidance molecules secretion by macrophages during bone resorption

Submitted to the
Austrian Marshall Plan Foundation

Bachelor's Program
„Medical and Pharmaceutical Biotechnology"
(0571)

by

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Area of emphasis/focus/special field:
Translational Medicine, Practical Training Semester at NYU School of Medicine
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Submitted on: February 12th, 2013

Statutory declaration

"I declare in lieu of an oath that I have written this bachelor thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This bachelor thesis has not been submitted elsewhere for examination purposes."

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Introduction

Osteoclasts and osteoblast are in constant communication which is essential for bone modeling and remodeling. Bone formation and resorption have to be at equilibrium to form a healthy bone [1,2]. If this equilibrium is disturbed in favor of bone resorption, pathological bone destruction takes place, a consequence of osteoporosis or rheumatoid arthritis, an inflammatory disease [3,4].

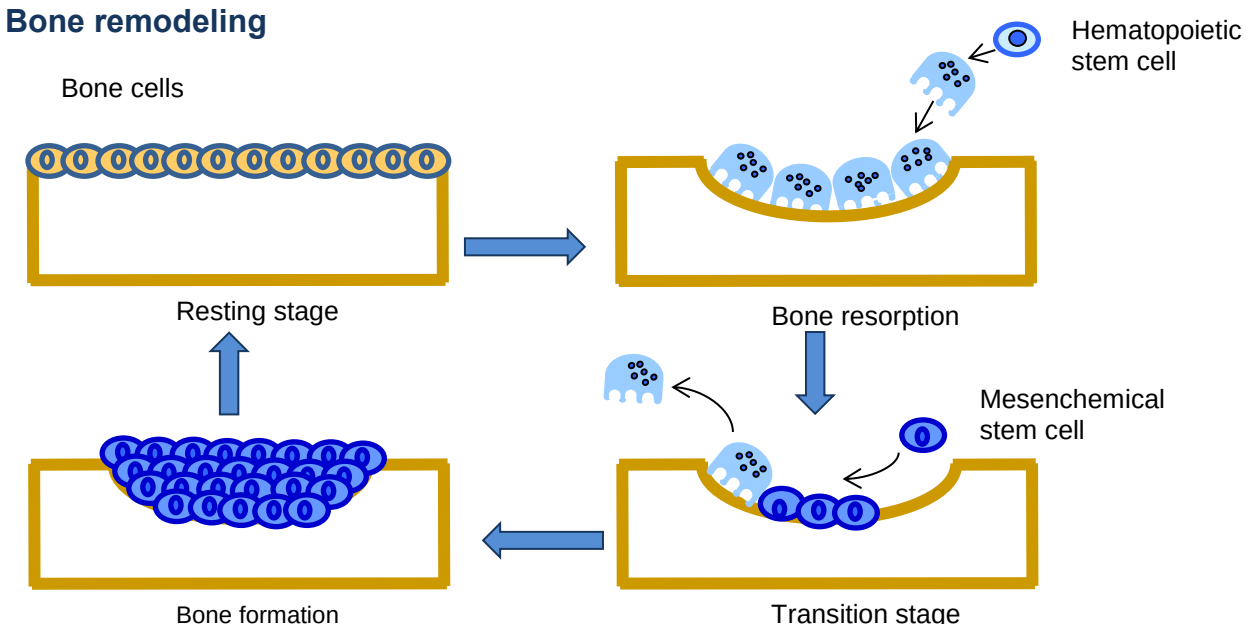

Figure 1 Schematic diagram of a bone remodeling system

Rheumatoid Arthritis (RA)

Rheumatoid Arthritis is one of the most common inflammatory arthritis worldwide. It is affecting between 0.5% to 1% of the population and it is predominant in women [17]. It is characterized by a chronic inflammation of the synovial cartilage and infiltration by blood-derived cells, T cells, macrophages, and plasma cells, which all show signs of activation [5][6]. This leads often to progressive destruction of joints and bone, which occurs after invasion of these tissues by the cellular synovial tissue [7].

Osteoporosis

Osteoporosis is the most common bone disease worldwide. The characteristics of the disease are low bone mass and structural deterioration of bone tissue, leading to bone fragility and increased vulnerability to fractures mainly of the hip, spine and wrist [16, 8].

Total hip arthroplasty

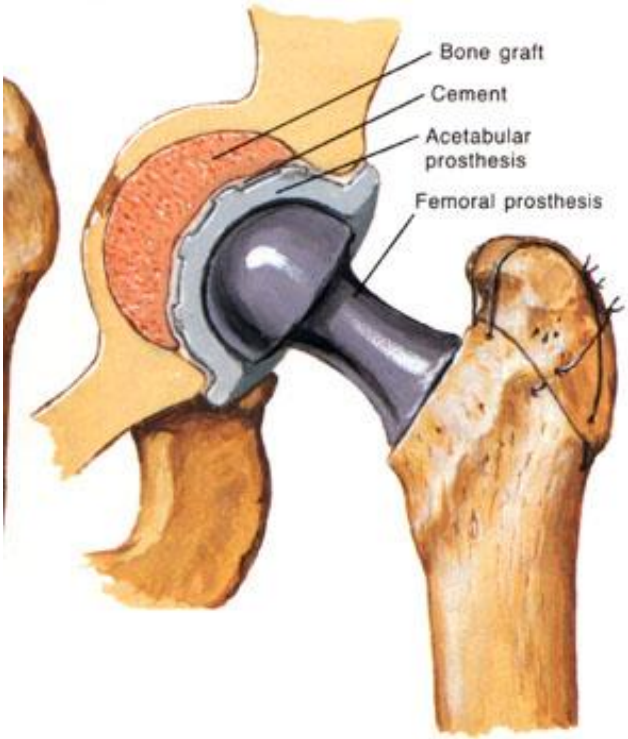

Figure 2 Diagram of total hip arthroplasty [14]

Total hip arthroplasty is one of the most clinically successful and cost effective interventions in health care. It reduces pain, improves the function and increases the quality of life in patients with debilitating hip disease [1]. But even with improving surgical techniques and implant design, the percentage of removal or replacement of the hip implant, called revision, has not decreased in the United States over the time [2]. To understand the cause of total hip athrotplasty failure and the types of revision total hip arthroplasty procedures being performed is crucial for increasing positive long-term patient outcomes [3].

One of the most common causes of prosthesis loosening is inflammation in response to wear particles near the prosthesis [4]. Wear particles are abrasions form joint and bone replacements of polymeric, metallic or ceramic source, that can animate the inflammatory-mediating cells and osteoclasts (cells that resorb bone) to the local site [9].

Macrophages

Macrophages and monocytes (their circulating precursor cells) are cells of the inborn immunity and are important effector cells for the elimination of microbes and particles. Phylogenetically they are the oldest agents of the innate immune reaction and even drosophila and plants have phagocyte –like cells. Activated Macrophages differentiate form blood circulating monocytes (Fig. 3). They secrete cytokines that activate phagocytes and stimulate the cellular reaction of the innate immune system, called inflammation. Osteoclast are differentiated Macrophages and perform bone resorption. Both reactions play an important role in bone erosion after prosthesis implantation following a total hip arthroplasty. [11]

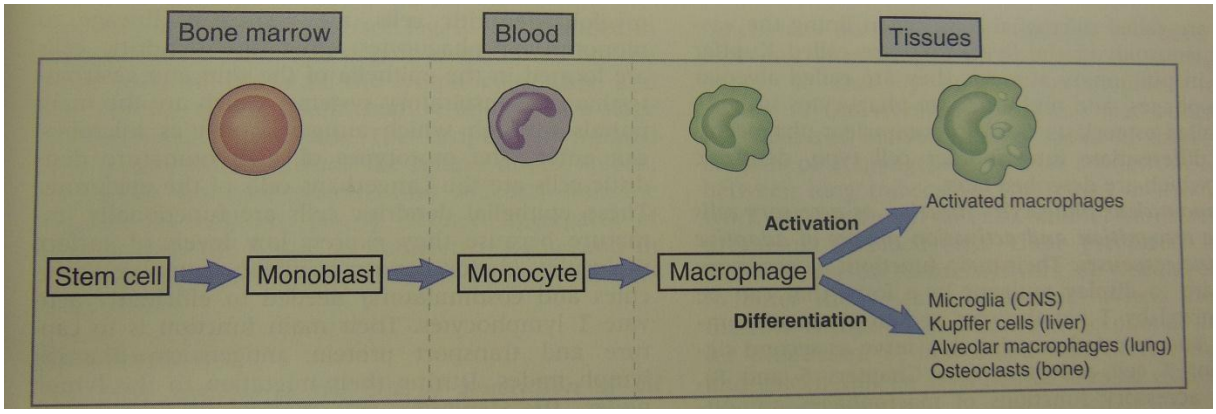

Figure 3 Maturation of mononuclear phagocytes [11]

If the particles cannot be destroyed by macrophages they cause chronically antigen stimulation and T cell and macrophage activation. This causes the particles surrounding by so called granulomas. The result is that the “spreading” is prevented, but it is also associated with severe functional impairment and it causes tissue necrosis and fibrosis. [11]

Adenosine and its receptors

Adenosine (Fig. 4) is a small molecule that is generated in the metabolism of adenine nucleotides is response to cellular stress like ischemia and hypoxia. It can modify a wide range of physiological processes by interacting with specific cell-surface receptors and it is also needed for the usual cell metabolism and growth.

Adenosine Receptors belong to the class of G protein-coupled receptors which play a role in inflammation upon activation via coupling to adenosine or its agonist and antagonists (Fig. 4)[6].

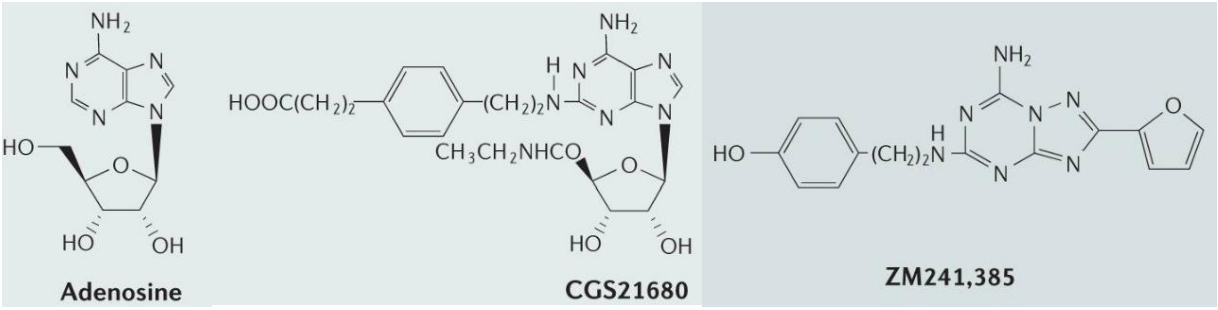

Figure 4 Molecular diagram of Adenosin, the A2A receptor agonist CGS21680 and its antagonist ZM241.385 [12]

Semaphorin 4D

The Axon guidance molecule Semaphorin 4D is expressed by macrophages and it was shown previously that it inhibits bone formation. A result of the binding of Sema4D to its receptor Plexin B1 on osteoblasts is the activation of the small GTPase RohA, which inhibits bone formation by suppressing insulin like growth factor 1 (IGF-1) signaling and by modulation osteoblast motility. [18]

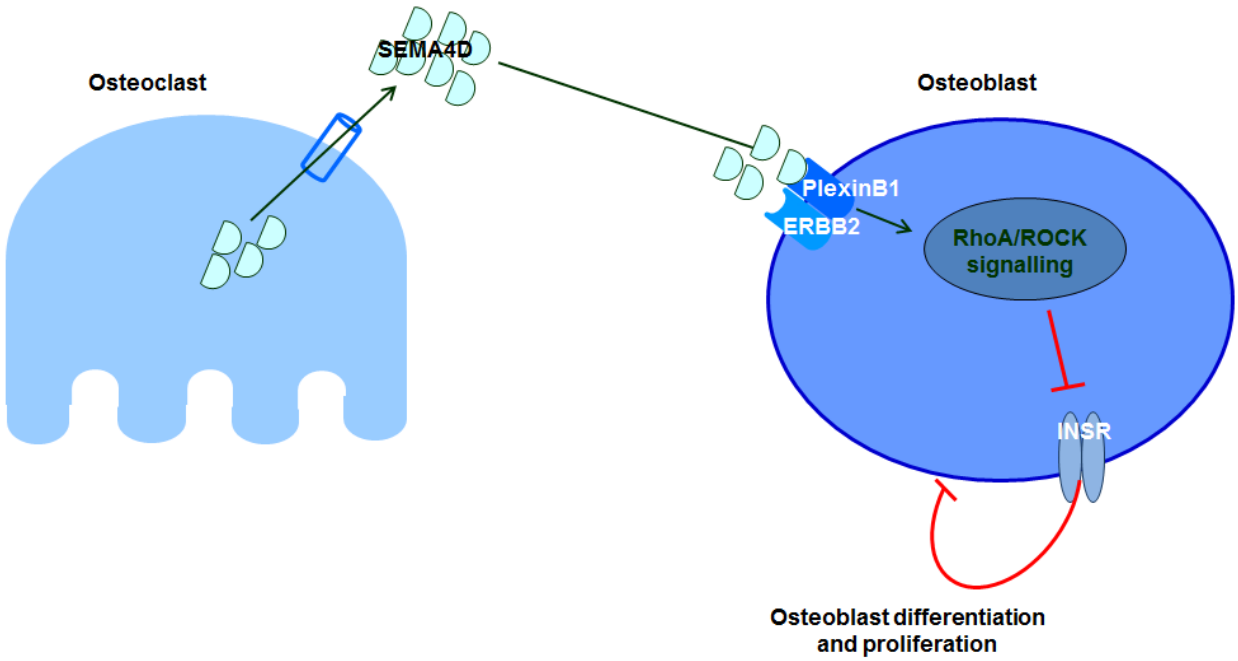

Figure 5 Semaphorin 4D secretion form osteoclast and role in osteoblast differentiation and proliferation [18]

Materials and Methods:

Animal work:

In the experiment a murine system is used. More concisely the C57BL/6J strain is used which has a black phenotype and is a wild type inbred stain.

Figure 6 Picture of a mouse from the C57BL/6J strain

The animals are injected with 1ml of 3% Thioglycolate into the peritoneum and after 5 days, the activated macrophages are collected from the mouse.

At first the mouse is sacrificed, then the fur on the lower part of the body is removed but the abdomen is still intact. Then 10ml PBS are injected into the peritoneum to flush out the activated macrophages. The PBS containing the activated macrophages is sucked out again and the cells are centrifuged for 10 min at 1400rpm and then resuspended in 10 ml medium and counted. According to the number of cells, $5 * 10^6$ cells are plated in cell culture dishes and filled up with the appropriate volume of medium.

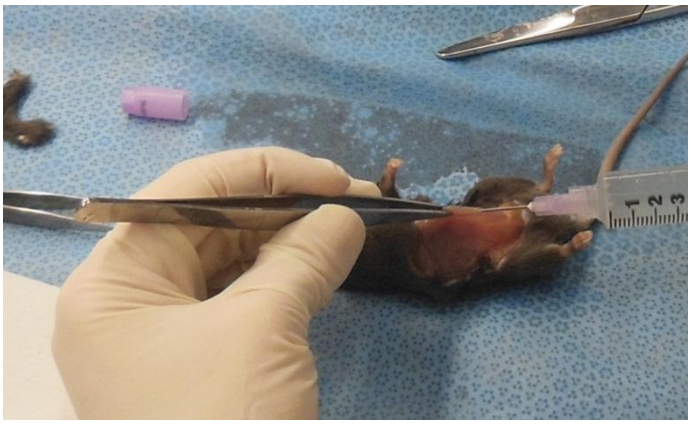
Figure 7 Picture of the extraction procedure

Cell Culture:

Cell treatment:

After 24 hours, a time course is done to determine the time where the cell treatment is most effective. The activated macrophages are treated with CGS, ZM and TNFα either for 12, 24 or 48 hours. A negative control is also done with DMSO.

Cell collection:

According to the different treating times, the activated macrophages are scraped into 500µL PBS as they do not react on trypsin. At first the medium is collected to see if the proteins are also expressed in the extracellular space. Then the cells are flushed with cold PBS to stop the cell metabolism. Then the cells are scraped into 0.5mL PBS and centrifuged at 13.000 rpm for 10 minutes and the supernatant is discarded. After that the collected medium and the pellets are stored at -80°C.

Protein extraction:

Out of Medium:

300µL of the collected medium is mixed with 900µL 70% ethanol, vortexed and stored over night at -80°C. On the next day, the mixture is centrifuged for 30 minutes at 13000rpm and 4°C. The supernatant is discarded and the pellet is resuspended in 100µL 1% SDS and then sonificated. The samples can be stored at -80°C.

Out of Cells:

The pellet is resuspended in 100-200µL RIPA buffer containing protease and phosphatase inhibitors (depending on size of the pellet). The mixture is then lysated with a 27G1/2 needle in a 1mL syringe. Then it is centrifuged for 20 minutes at 4°C and maximum speed. The supernatant is then transferred into a new tube and the pellet is discarded. The samples can be stored at -80°C.

Protein Quantification

The protein concentration is determined via Bicinchoninic acid assay (BCA). A standard curve is generated with known concentrations of Bovine Serum Albumin (BSA) (concentrations are: 250µg/mL, 500µg/mL, 750µg/mL, 1000µg/mL, 1500µg/mL, 2000µg/mL).

Then 10µL of each sample are mixed with 1mL of BCA working solution and the samples are incubated for 30min at 37ºC. After that the protein concentration is determined with the spectrophotometer.

Western Blot

Table 1 Composition of the 10x Running buffer

10x Running Buffer for 1 Liter	
Tris Base	30.3g
Glycine	144.2g
Distilled water	Fill it up to 1 Liter

Table 2 Composition of the 1x Electrophoresis buffer

1x Electrophoresis Buffer for 1 Liter	
10x Running Buffer	100mL
10% SDS	10mL
Distilled Water	890mL

Table 3 Composition of the lower gel, according to LMW or HMW proteins

ingredients	for separation of proteins with LMW: 10% gel	for separation of proteins with HMW: 7.5% gel
1.5M Tris pH8.8	2.5mL	2.5mL
Distilled Water	4mL	4.8mL
30% Acryl amide	3.3mL	2.5mL
10% SDS	100µL	100µL
10% APS	100µL	100µL
TEMED	8µL	8µL

Table 4 Composition of the upper gel

ingredients	for stacking of the proteins
0.5M Tris pH8.8	1.25mL
Distilled Water	3.2mL
30% Acryl amide	0.5mL
10% SDS	50µL
10% APS	50µL
TEMED	5µL

According to the protein concentration the samples are prepared so 50µg of protein are in each well of the SDS page. Then the amount that equals one quarter of the protein concentration of 4x the loading buffer is added to the sample, so that the end concentration of loading buffer in the samples is 1x. After that, the samples are spinned down and then cooked for 5min. The samples can be stored on ice till the gels are ready for loading.

Then a SDS PAGE is done. All ingredients for the gels are mixed and bubbles are avoided. The gels are prepared between appropriate glass plates in chambers.

Attention has to be paid, when the 10% APS and the TEMED are added, at the end of the preparation, as these two are the polymerization reagents.

At first the lower gel is put in between the glass plates and overlaid with distilled water, as the gel can only polymerize when no oxygen is present. When the lower gel is solid, the water is poured out and the second gel is added on top of the lower gel. The appropriate comb is put into the liquid gel according to the number of samples to be loaded. When the second gel is solid the comb is removed, the glass plates are put into the electrophoresis chamber and the inner space is overlaid with 1x running buffer.
After the marker is loaded into the first well, the samples are loaded onto the gel. And the electrophoresis is run at 150V for 1 hour.

Table 5 Composition of the Nitrocellulose or Transfer buffer

1x Nitrocelluose Buffer/Transfer buffer for 1 Liter	
10x Running buffer	100mL
Methanol	200mL
Distilled water	700mL

Now the separated proteins are transferred from the gel onto a membrane. The wet method and the nitrocellulose membrane are used. At first the stiff paper, the membrane and the sponges are soaked with transfer buffer. Then they are put into the western blot holders in the following order: Sponge, 2 stiff papers, membrane, gel, 2 stiff papers, and another sponge. The sandwich is put into the black and white holder and it is made sure, that the gel faces the black side of the holder. Then the holder is put into the transfer chamber and it is made sure, that the black side of the holder faces the black side of the camber. Cooling thermal packs are added to the chamber. And then the whole chamber is filled with transfer buffer.

The important step is, that no bubbles are between the gel and the membrane and the membrane to face the anode. The chamber is closed with the lid and connected to the power source and the western blot is run for 1 hour at 90V in the fridge, because the power generates heat.

Table 6 Composition of the TBST

TBST (Tris Buffer Saline Tween 20)	
5M NaCl	30mL
1M Tris pH 7.4	20mL
Tween	1mL
Distilled water	949mL

Table 7 Composition of the Blocking buffer

Blocking buffer	
Non-fat dry milk powder	5g
100mL	TBST

When the transfer is finished, the nitrocellulose membrane is taken out of the chamber and the gel is thrown away. The nitrocellulose is cut in the right form, and one corner is cut, so it is easier to keep track of the orientation of the membrane. Then the membrane is put into a small clean box and the membrane is overlaid with the blocking buffer. The membrane is incubated for 1 hour at room temperature under agitation. The primary antibody is diluted according to the recommended dilution factor stated in the antibody datasheet. The used dilution solution is TBST plus 2% non-fat milk. Common dilutions for a goat or mouse primary antibodies are 1:1000 or 1:2000. After the blocking, the membrane is incubated over night at 4°C and agitation in the primary antibody. On the next day, the primary antibody can be collected and stored for reuse at -20°C. It can be reused up to 2 times. The membrane is then washed 3 times for 10 minutes with TBST under fast agitation, to remove the primary antibody. The TBST is changed between each washing step.

The secondary antibody is diluted in TBST. Ratios are between 1:1000-1:10.000, as the datasheet recommends. The secondary antibody is incubated for 1 hour and slow agitation at room temperature and cannot be reused. The secondary antibody is drained and the membrane is washed again 3 times for 10 minutes with TBST to remove the secondary antibody. The TBST is changed between each washing step.

Before the image is taken, the membrane has to be incubated with the ECF, to cleave the Phosphate of the AP and to create a fluorescent signal. The membrane is placed facing the proteins upwards on the glass plate of the Typhoon Trio Imager, and 1mL of ECF is equally distributed on the membrane. Then the lid of the Imager is closed and the membrane is incubated in the dark for five minutes.

After the five minutes incubation, the ECF is drained and the membrane is placed on the left corner of the glass plate of the Imager facing the proteins downwards.

During the incubation time, the appropriate settings in the scanning program are adjusted. Fluorescence and excitation wavelength of 488nm according to the used antibody is taken. Also the orientation of the image has to be adjusted to mirror-inverted.

After all the precautions are taken, the scanning can be started, and the file is stored under the appropriate name on the NYU server.

After about five minutes, the scanning procedure is completed, and the image can be observed.

PCR

PCR is a method for amplifying defined DNA fragments. It can thus be used to check for gene expression in the cells. But for this task cDNA of a cell is needed. Therefore a RNA Extraction and a reverse transcriptase have to be performed.

RNA Extraction

It is important, that everything is cleaned with RNaseZap Wipes.

Depending on the number of cells in the sample, (should be 5.10^6 as this number of cells was plated) the pellet is resuspended in 350µL RLT buffer and vortexed. Then the mixture is transferred into the QIAshredder columns for homogenation and centrifuged for 2 minutes at high speed. The column is discarded and 350µL 70% Ethanol are added to the tube and mixed with the pipette. This mixture is then transferred into the RNeasy column and the column is linked to a 2mL collector tube and centrifuged 15seconds at 10000rmp. The elute is discarded. The collector tube is reused and another 350µL RW1 buffer are added to the RNeasy column and centrifuged for 15 seconds at 10000rpm. The elute is again discarded. Once again the collector tube is reused. 10µL DNase are mixed by inversion with 70µL RDD buffer, put into the RNeasy column and incubated at roomtemperature for 15 minutes. After that, 350mL of RW1 buffer are added to the RNeasy column and again centrifuged for 15 seconds at 10000rpm and the elute is discarded.

The RNeasy column is now transferred to a new collector tube, and 500µL RPE buffer are put onto the column and it is centrifuged for 15 seconds at 10000rpm. The elute is discarded and the step is repeated. 500µL RPE buffer are added to the column and at first centrifuged for 2 minutes at 10000rpm and then for 1 minute at 13000rpm and the elutes are discarded both times.

To elute the RNA out of the column, the column is transferred to a 1.5mL tube and 30-50µL RNase free water are pipetted onto the filter and the tube is centrifuged for 1

minute at 10000rpm. To concentrate the sample, the elute is again added to the filter and centrifuged for 1 minute at 10000rpm.

RNA Quantification

The RNA is quantificated with a spectrophotometer. The machine is blanked with water. 2µL of the RNA sample are mixed with 48µL RNase free water. In the RNA/DNA section of the program, the RNA is selected (1 A260nm=40µg RNA). After each sample the cuvette is washed with water. At the end, the results are printed.

Another method is the NanoDrop where 1µL of the elute is put onto the metal pedestal and the concentration is measured.

Figure 8 Picture of the nanodrop method [15]

RT – Reversed Transcriptase

RT is done to get the c-DNA out of the RNA to perform a PCR. According to the RNA concentration the appropriate amount of RNA is used to do a RT.

A master mix is done. Therefore Magnesium Chloride, 10x PCR buffer, dATP, DTTP, dGTP- dCTP, RNA inhibitors, random Hexamers and multiscribe reverse are mixed and stored on ice. They are never defrosted. Then the extracted RNA the water and the mastermix are mixed. Then the RT is run with the appropriate program. The Reverse Transcribe step is 15 minutes at 42°C the Denature step is 5 minutes at 99°C and the cooking step is 5 minutes at 5°C. The cDNA can be stored at -20°C.

PCR – Polymerization Cain Reaction

The PCR is done after the cDNA is gained out of the RT. The mastermix is prepared according to how many samples and genes to be detected. Due to pipetting errors, it is recommended to prepare for one more sample to prevent lack of the mastermix. One mastermix is prepared for each gene to be detected. The Brilliant III Ultra-Falst SYBR Green QPCR master mix was used and it is complete provided. The two primers, forward and reverse (individually designed for each detected gene) and the water are added to the mix and then 20µL of the mix and 5 µL of the sample are pipetted into a PCR tube.

The PCR machine used is the eppendor realplex2 Mastercycler epgradientS. The principle of the cycles is dicribed beneath.

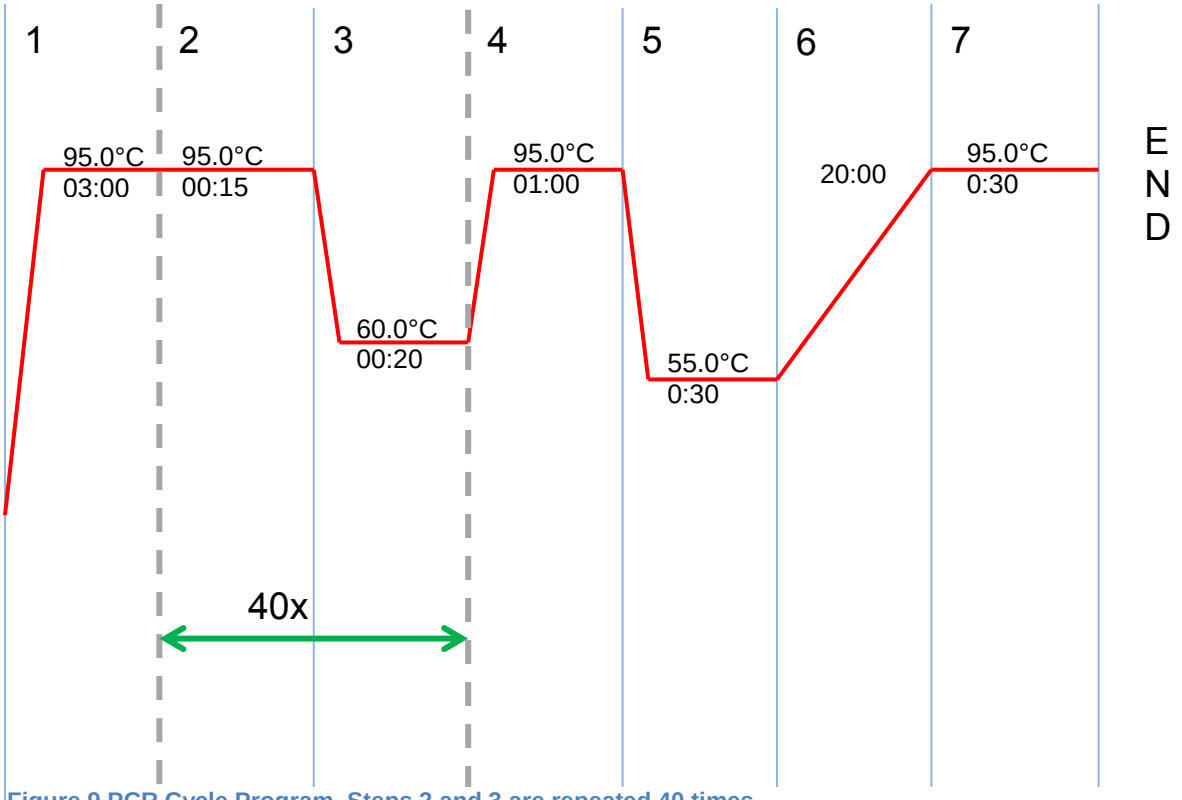

Figure 9 PCR Cycle Program. Steps 2 and 3 are repeated 40 times.

The concentration values of SyBr Green in the end give information whether the genes are expressed in the sample or not. The results are then confirmed with a agarose gel.

IHC

Immunohistochemistry is a method to analyze slides that are previously obtained from the wear particle induced model. The calvaria from sacrificed mice is fixed in 4% paraformaldehyde and 48 hours later, the calvaria is started to be decalcified in 10% EDTA. The EDTA is changed every day for four weeks and at the end washed with dH2O for three hours. After that they are incubated in 70% ethanol overnight and the following day they are incubated in 80% ethanol for two hours and then two hours in 90% ethanol and again over night in 100% ethanol overnight. On the next day they are given to another laboratory in which the calvaria is embedded in paraffin and then cut and fixed on slides.

The slides are returned to our laboratory and the actual IHC procedure starts. At first the pre-deparaffinization is done. Therefore the slides are put into a heater and are kept there for 30 minutes at 60°C. Then the deparaffinization is done, where the slides are 3 times put into xylene for 10 minutes, which can be reused till it seems dirty. This steps have to be done under the hood, as xylene develops a strong smell. During the hydration steps the slides are at first put into 100% ethanol, then into 90% ethanol, then 80% ethanol and then 70% ethanol for each two minutes and at the end five minutes into distilled water.

Table 8 TE Buffer composition

TE Buffer (50mM Tris Base, 1mM EDTA, 0,5% Triton X-100, pH 8.0)	
Tris Base	6.1g
EDTA	0.37g
Triton X-100	5mL
Distilled water	1000mL

Table 9 Proteinase Stock Solution composition

Proteinase K Stock Solution (20x, 400µg/mL or 12 units/mL)	
Proteinase K (30 units/mg)	0.008g
TE Buffer, ph 8.0	10mL
Glycerol	10mL

Table 10 Working solution composition

Working Solution (1x, 20µg/mL or 0.6 units/mL)	
Proteinase K Stock Solution	1mL
TE Buffer, ph 8.0	19mL

The pH from the TE Buffer is adjusted by HCl and stored at room temperature. The Proteinase K stock solution can be stored in aliquots at -20°C for 2-3 years.

The Antigen Retrieval is done in Proteinase K working solution by covering the slides in it and incubating them for 15 min at 37°C in the water bath. The sections are cooled down but precautions have to be taken that they do not dry. Then they are rinsed two times with PBS for two minutes. Then the internal peroxidase removal is

done to reduce the background noise. Therefore 3% H_2O_2 in Methanol is put onto the slides and they are incubated for 15 minutes at room temperature. Then the slides are again rinsed with 3% PBS-BSA.

Table 11 Blocking Solution

Blocking Solution	
3%PBS-BSA	9.4mL
TritonX-100	0.1mL
FBS 5%	0.5ML

The blocking is done with blocking solution which is incubated at room temperature for one hour. The slides are covered in the solution and then with a plastic coverslips to prevent them from drying.

Then the primary antibody is put in the appropriate dilution (Semaphorin 4D in 1:250 dilution) onto the slides and then they are covered with plastic coverslips to prevent them again from drying. The slides are then stored overnight in a humidified camber at 4°C.

On the next day, the plastic slips are removed and the solution is drained. The slides are washed 3 times with 3% PBS-BSA and then the secondary antibody is put in a 1:200 dilution in 3%PBS-BSA on the slides and incubated for 1 hour at room temperature. Plastic coverslips prevent again the drying. The slips are removed and the solution is drained and the slides are washed once with 3%PBS-BSA and then two times in PBS. The signal has to be developed. Therefore Fast 3'3'-Diaminobenzidine prepared according to the manufacturer's protocol and the slides are covered with it for 3 minutes until the color shows. The solution is drained and the slides are washed three times with PBS. The counteract is done with hematoxiline for 1 minute and then the slides are washed in distilled water until the water gets trans lucid.

The diaminobenzidine reacts with horseradish peroxidase and the enzyme conjugated to the antibody- antigen substrate produces a brown colored precipitate. The slides are mounted with Permount mounting medium and the images were observed under light microscope (Nikon) equipped with NIS-Element s F3.0 SP7 software and under a Leica microscope equipped with SlidePath Digital Image Hub version 3.0 software. The slides is analyzed by counting the brown colored positive cells on the scanned IHC slides by Leica SCN400F viewed on the software Digital Image Hub.

Results

The experiments are performed to identify the consequences of treatment of different cells with A2A agonists and antagonists. Especially the semaphorin 4D secretion is observed. At first the expression of semaphorin 4D and Adenosine 2A receptor is performed. At the same time the expression of some other axon guidance molecules and the other adenosine recepors is checked.

Axon guidance molecules in Macrophages

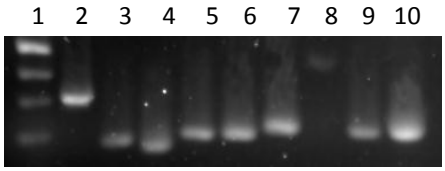

Figure 10 Axon guidance molecule expression in macrophages from mice

1 Gene DNA PCR ladder 100bp
2 Ephrin B1
3 Ephrin B1 receptor
4 Ephrin B2 receptor
5 Ephrin B3 receptor
6 Semaphorin 3A
7 Semaphorin 4D
8 Plexin A1
9 Plexin B1
10 Neurophilin

Axon guidance molecules in RAW

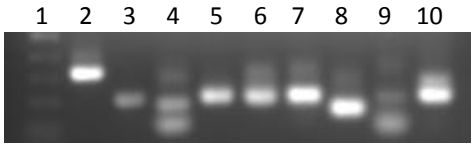

Figure 11 Axon guidance molecule expression in RAW cells

Adenosine receptors

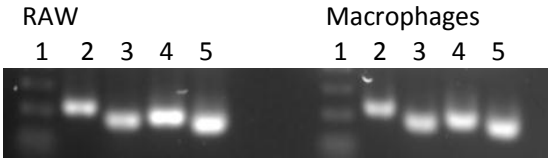

Figure 12 Adenosine receptor expression in RAW cells and macrophages form mice

1 Gene DNA PCR ladder 100bp
2 A1 Receptor
3 A2A Receptor
4 A2B Receptor
5 A3 Receptor

The PCR Products show, that all searched axon guidance molecules are expressed in mouse macrophages and RAW cells. Especially semaphorin 4D which the project is about can be seen in lane 7 in figure 14 and 15.

It can also be seen, that all Adenosine receptors are expressed in mouse macrophages and RAW cells. Adenosine 2A receptor can be seen in both lane 3s in figure 15.

The IHC is performed together with the supervisor Aranzazu Mediero. The mouse calvaria is given to the Histology core at NYU School of Medicine and then the slides are stained.

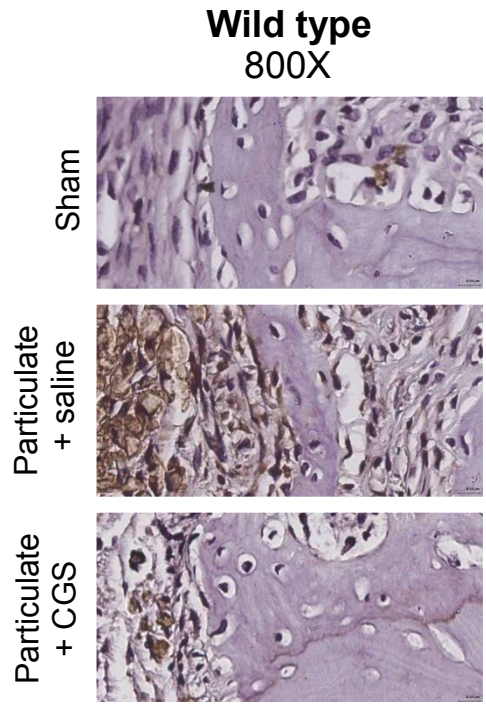

Figure 133 Changes in Semaphorin 4D expression due to Adenosine 2A receptor activation in Wild type mice

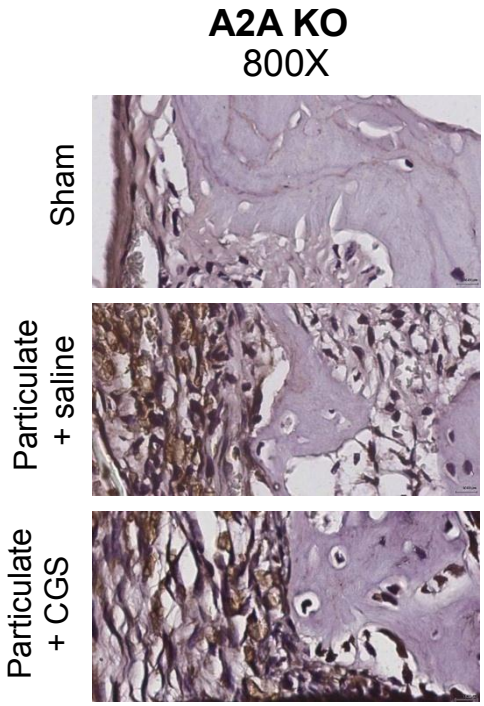

Figure 14 Changes is Semaphorin 4D expression due to Adenosine 2A receptor activation in A2A knock out mice

The Immunohistochemistry is performed with semaphorin 4D antibody, visible as the brown color in the images.

The wild type slides show, that the dark inflammation area is the biggest with the wear particle and saline treatment, whereas the dark inflammation area with the wear particle treatment and CGS is the same than with the PBS treatment called Sham.

The A2A knockout mice slides show, the same inflammation area in the CGS and particle treated and the saline and particle treated. The less inflammation area can be observed in the PBS treated called Sham slides.

Discussion

To show how Adenosine 2A receptor stimulation affects semaphorin 4D secretion, the axon guidance molecules and the A2A receptor expression have to be shown in the different cell types. This is done via PCR. Figure 10 to 12 show that the sought semaphorin 4D as well as other axon guidance molecules and the A2A receptor are expressed in mouse macrophages and RAW cells. With this knowledge the other experiments can be started.

In the immunohistochemistry it is shown that semaphorin 4D is highly present in the dark inflammation area in the mouse calvaria, which means that high amounts of semaphorin 4D are secreted from macrophages. This secretion is modified by CGS an A2A receptor agonist.

It was previously shown, that semaphorin 4D mediates in osteoclast osteoblast communication. It was demonstrated that an antibody against semaphorin 4D enhances osteoblastic bone formation in ovariectomized osteoporotic mice. The semaphorin 4D secreted by osteoclast and macrophages balances the bone resorption and suggests semaphorin 4D as new therapeutic target in Osteoporisis. [18]

This could mean, that if semaphorin 4D secretion is suppressed, osteoblastic bone formation is enhanced.

References

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